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*He believed that a meritorious discovery may fail of appreciation because of the faulty manner in which it is announced to the world, and that an editor may be of service to an investigator. He believed that a scientific journal, the organ of a national science, should be characterized by scientific merit, rhetorical excellence, and prompt publication of its contributions, together with typography and illustrations that are pleasing to the eye. These ideals he has maintained.*

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to William Townsend Porter

# A CONCEPT OF MEAN ALVEOLAR AIR AND THE VENTILATION—BLOODFLOW RELATIONSHIPS DURING PULMONARY GAS EXCHANGE<sup>1</sup>

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IN THE past many different methods have been employed for the direct sampling of alveolar air. A sound criticism of the validity of the technique has usually been futile since one was at loss to define or determine mean alveolar composition. The ease with which alveolar air composition can be altered by the slightest change in ventilation is very impressive and makes one aware of the possibility of unequal ventilation in various parts of the lung and the consequential regional variability of gas concentration. The concept of unequal bloodflow to the various alveoli has received less attention, although this factor is equally important in altering the gas composition.

It is the purpose of this paper 1) to define the alveolar air composition in terms of alveolar ventilation and pulmonary bloodflow which allow one to define a concept of mean alveolar gas composition; 2) to discuss a method for the direct sampling of mean alveolar air; 3) to compare this with the Haldane technique of sampling alveolar air; 4) to predict on the basis of the ventilation-bloodflow equations the effect of unequal ventilation and bloodflow upon the alveolar-arterial oxygen gradient.

*A Concept of Mean Alveolar Air.* The alveolar air equation and the alveolar ventilation equation have given us a theoretically precise definition of the relation of the alveolar-gas concentrations and the ventilation (1). When this equation is combined with the Fick equation, it allows one to express the alveolar gas concentration in terms of bloodflow and ventilation (2).

Let

$F$  = bloodflow in liters/min.

$V_a$  = alveolar ventilation in liters/min. B.T.P.S.

$(A-V)O_2$  = arterial-venous oxygen difference in ml/l.

$X_0$  = oxygen intake in ml/min. S.T.P.

$Q$  = respiratory quotient

$pC$  = partial pressure of  $CO_2$  in alveolar air.

then the bloodflow according to the equation of Fick is

$$X_0 = F(A-V)O_2 \quad (1)$$

and the alveolar ventilation according to Fenn *et al.* (1) is

$$X_0 = \frac{V_a \times pC}{.864Q} \quad (2)$$

Combining the equations and eliminating  $X_0$  we have

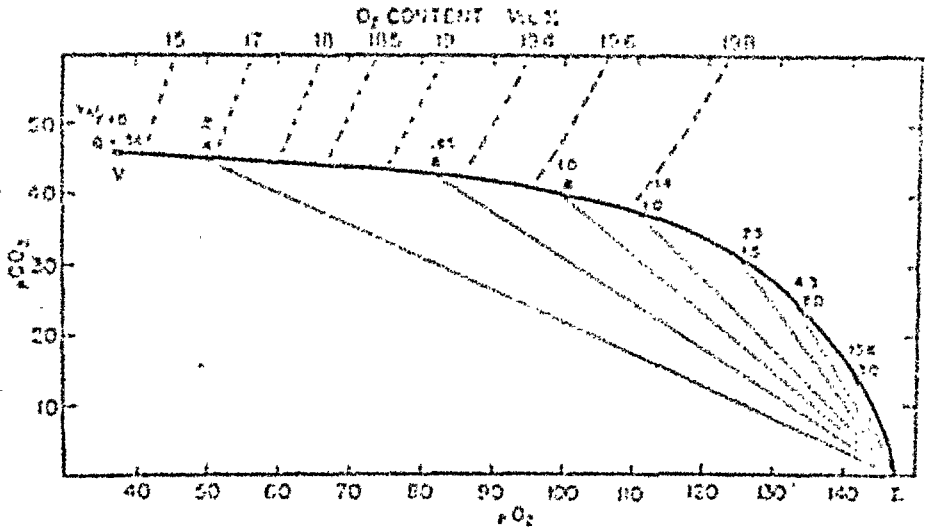
$$pC = \frac{F}{V_a}(A-V)O_2(.864Q) \quad (3)$$

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<sup>1</sup> Work done under contract with the Air Materiel Command, Wright Field.

and  $p\text{CO}_2$  tensions which satisfy equation 3.

This is done in the following manner. Various gas R.Q. lines are plotted radiating from the inspired  $\text{O}_2$  tension, I, on the Penn  $\text{O}_2$  -  $\text{CO}_2$  diagram (fig. 1). The point describing the  $\text{O}_2$  and  $\text{CO}_2$



We have now defined for this specific example all the possible simultaneous  $O_2$  and  $CO_2$  tensions which could exist in any part of the lung as well as in the pulmonary capillary blood if we assume that the terminal diffusion gradient across the alveolar membrane is for practical purposes negligible. The next object is to determine the *mean alveolar* gas concentration. This is done easily, if the R.Q. is known. Thus, if we choose in our example an R.Q. of .8 then the intersection of this gas R.Q. line with the curve of figure 1 determines the only point at which blood can exchange at this particular R.Q. This is at a  $pCO_2$  and  $pO_2$  of 40 and 100 mm. Hg respectively and assumes that all blood in all the alveoli is exchanging at this particular R.Q. Of course, it is very probable that this is not normally the case and that alveoli exchange individually at various R.Q.'s and that only the mean of all of them result in an R.Q. of .8. As will be discussed below, a normally distributed population of  $V_a/F$  values will not appreciably affect the predicted alveolar value, but only change the *arterial*  $pO_2$ .

The validity of the theoretical alveolar air curve (fig. 1) should be experimentally demonstrable if one could alter the  $V_a/F$  ratio without changing the steady state and the venous tension,  $V$ . The closest approach to such a test can be accomplished by analysis of the alveolar air composition after breath holding and hyperventilation when this is done at various intervals up to one circulation time (20 sec.). It might be assumed that the venous tension will remain constant during this period. Such experiments have been previously described (4), and are here compared with the theoretical curve (fig. 2). Although a discrepancy is apparent, probably due to altered  $CO_2$  output, cardiac output and partial recirculation, a general agreement is rather striking and lends support to the theoretical conclusions regarding the possible combinations of  $CO_2$  and  $O_2$  which could exist simultaneously in the alveoli. It has always been an impressive fact that the simultaneous gas tensions could be altered only along a very narrow and predictable pathway no matter by what method the steady state was altered (5).

*Sampling of Mean Alveolar Air.* It has been shown above that the mean alveolar air can be theoretically defined provided 3 facts are known: 1) the venous gas tensions, 2) the inspired gas tensions and 3) the R.Q. (It also presupposes that there exists no terminal alveolar membrane gradient.) The question now arises how close these values for the theoretical mean alveolar air can be approached by direct methods which do not involve the sampling of mixed venous blood.

Riley *et al.* (6) have approached this problem by collecting and analyzing the arterial blood for the  $CO_2$  tension. Since such a sample represents a mixture of practically all the blood that has passed through the pulmonary capillaries, it must represent the mean arterial  $pCO_2$  tension and is for all practical purposes in equilibrium with the alveolar  $pCO_2$ . (It can be shown that a considerable venous admixture has a negligible effect upon the mean arterial  $pCO_2$  tension. Furthermore, it is shown below that a normal probability distribution of  $V_a/F$  among the alveoli likewise has a negligible effect.)

On the basis of the theoretical discussion above, this mean arterial  $pCO_2$  value should be identical with the mean alveolar  $CO_2$  tension. (This theoretical mean tension has been designated as 'ideal' tension by Riley (3)). Thus, substituting mean alveolar  $CO_2$  for mean arterial  $pCO_2$  and determining the respiratory quotient from the expired air, Riley is able to calculate the *mean alveolar*  $pO_2$  from the alveolar air equation. This method is theoretically entirely sound for obtaining the mean alveolar composition but offers practical difficulties since it requires an arterial puncture and considerable skill in the determination of arterial  $pCO_2$ . Furthermore, any single determination of arterial  $pCO_2$  by the method of Riley *et al.* (6) can only be read with an accuracy of  $\pm 3$  mm.  $pCO_2$ .

On the other hand, methods of obtaining alveolar air directly offer many practical advantages and it is our purpose to ascertain how closely these methods approach the theoretical concept of mean alveolar air. In the past there have been essentially 2 methods of sampling alveolar air. In the original method (Haldane) all the expiratory reserve was forced out of the lung and the last part analyzed, while others have sampled from the last part of each normal tidal volume. These 2 methods do not yield identical results and the difference can probably be best appreciated when the time course of  $\text{CO}_2$  concentration is recorded for each individual breath on a fast infrared  $\text{CO}_2$  analyzer recently described by Fowler (7) from this laboratory (fig. 3). It can be seen that after the deadspace is washed out with a normal tidal expiration, the  $\text{CO}_2$  concentration rises quickly and tends to plateau off. With a forced expiration (Haldane), however, the alveolar  $\text{CO}_2$  keeps on rising. This curve is typical of some 15 healthy individuals who have been recorded by this method.

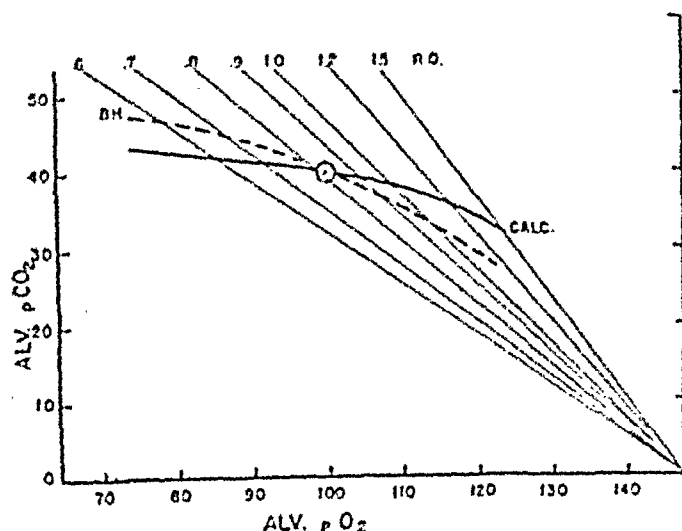


Fig. 2. THEORETICAL ALVEOLAR AIR COMPOSITION CURVE (solid line) compared with directly obtained samples (dashed line) when the  $V_a/F$  ratio of the normal alveolar air (open circle) is altered by breath holding or hyperventilation. (see text).

With the Haldane technique part 'X' is sampled. In a method described by us recently (5, 8), part 'Y' is sampled. In this example the  $\text{CO}_2$  difference between these 2 points is approximately 3 mm. Hg.

Sampling the last part of each normal tidal has several advantages, for it averages the last 10-15 cc. of each tidal volume expired during normal breathing and, furthermore, it does not require trained subjects for delivering a properly timed, forced expiration. In addition to the automatic sampling the continuous analysis is extremely convenient. In order to determine the reliability of these samples as representing mean alveolar air, tests were performed comparing the alveolar  $\text{pCO}_2$  with the arterial  $\text{pCO}_2$ .

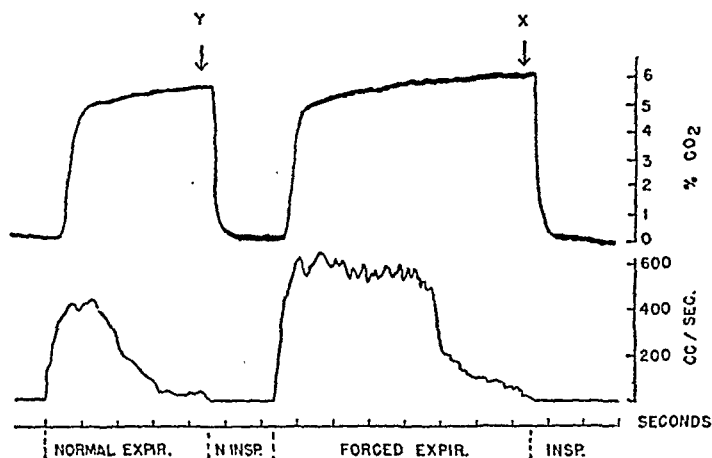
The  $\text{CO}_2$  comparisons were carried out by Miss Suskind in this laboratory (unpublished). While the alveolar air was analyzed continuously by the method referred to above, blood samples were taken from the radial artery in the human subjects and the femoral artery in the dogs. This approach allowed for simultaneous sampling of these 2 components. Nine experiments were carried out on man and 80 were done on dogs under nembutal or pentothal narcosis. The mean difference

for all experiments between the alveolar  $p\text{CO}_2$  and the arterial  $p\text{CO}_2$  was 0.78 mm. Hg, standard error of the mean 0.58. (In our hands we find that on the average the  $p\text{CO}_2$  determination of the blood differs from the equilibrating test gas mixture by less than .3 mm. Hg with a standard deviation of 2.5.) These observations would suggest that the alveolar  $p\text{CO}_2$  obtained by this method of sampling from the last fraction of each tidal volume is in very close agreement with the arterial  $p\text{CO}_2$ . Therefore, this value, as pointed out above, represents the mean alveolar  $p\text{CO}_2$  unless an appreciable terminal membrane gradient exists, which is unlikely (6).

In the continuous method of alveolar air analysis the  $p\text{O}_2$  is simultaneously analyzed and must, therefore, represent the mean alveolar  $p\text{O}_2$  provided that the alveolar air R.Q. is identical to the expired air R.Q., the latter representing the classical standard.

To test the possible discrepancy between these, the alveolar R.Q. was determined by continuous alveolar air analysis and compared with R.Q. values from the

Fig. 3. INSTANTANEOUS  $\text{CO}_2$  CONCENTRATION recorded by an infrared analyzer during a normal expiration and a forced expiration. The simultaneous expiratory velocities are recorded below. The inspiratory velocity curve is not shown. X and Y denote the part of the alveolar air sampled by the Haldane technique and the continuous technique, respectively.



collected expired air. The expired air was analyzed by the same automatic meters as well as by Haldane gas analyses in 27 experiments on 7 different subjects. The average values are presented in table 1 A and show no significant difference between the expired air and the simultaneous alveolar air R.Q. These results would suggest that this method of sampling yields an R.Q. which is in agreement with expired air R.Q. values. Thus, we may draw the conclusion that the alveolar air sampled continuously from the end of normal tidal expirations, yields a value which must be very close to the theoretical mean alveolar  $p\text{O}_2$  and  $p\text{CO}_2$ .

The other method (Haldane) for sampling alveolar air must also be discussed. This method analyzes the last part of the forced expiration. When this method is compared with samples taken by the previous method (8) one finds on the average a 2 mm. higher  $\text{CO}_2$  and a 5 mm. lower  $p\text{O}_2$  for the Haldane technique. This is not surprising when we look at figure 2 and also consider that this forced expiration usually lasts 2-3 seconds beyond the time taken for a normal expiration (end-expiratory sample). Similar differences were obtained by Riley *et al.* (6) who compared the alveolar  $\text{CO}_2$  obtained by the Haldane technique (end-inspiratory samples) with the arterial  $p\text{CO}_2$ . They observed on the average a 4.4 mm. Hg higher value in the alveolar sample taken at rest.

It should be pointed out that the subjective impression of the time it takes to expire completely may be quite misleading, unless a simultaneous air velocity record is obtained. It is during the last phase of expiration that the  $\text{CO}_2$  in many subjects rises rather steeply due to the smaller lung volume which is still exchanging with the blood. Actually, tests have shown (unpublished) that during this prolonged expiration, the oxygen uptake remains normal, but the  $\text{CO}_2$  output is reduced. The net result is that not only must the  $\text{CO}_2$  rise above and the  $\text{O}_2$  fall below the average value existing at the end of a normal tidal, but the ratio between oxygen uptake and  $\text{CO}_2$  output is altered, producing an abnormally low R.Q. These changes become exaggerated when larger intervals than 2-3 seconds are used for the forced expiration or when the breath is held (4).

Table 1 B shows the alveolar R.Q. differences obtained by the 2 methods of sampling. These experiments were done on 10 subjects and were reported elsewhere (8). But in view of the values cited in table 1 A, they assume new interest

TABLE 1

A COMPARISON OF EXPIRED AIR R.Q. AND ALVEOLAR AIR R.Q. (END OF NORMAL TIDAL SAMPLE)				B ALVEOLAR R.Q. OBTAINED BY SAMPLES FROM:			
	Mean R.Q.	S.E.M.	n		Mean R.Q.	S.E.M.	n
Expired air R.Q.....	.828	.010	27	End of normal tidal ex- piration.....	.821	.0052	148
Alveolar air R.Q.....	.829	.007	27	End of forced expiration....	.790	.0054	148
$\Delta$ .....	.001			$\Delta$ .....	.031		

since they show the Haldane method to deviate from the expired air R.Q. and by an average value of .03 R.Q. units. This would support the contention that a forced expiration is actually equivalent to breath holding which brings about similar changes in  $\text{O}_2$ ,  $\text{CO}_2$  and R.Q. of alveolar air (4).

*The  $\text{O}_2$  and  $\text{CO}_2$  Dead Space.* The discrepancy between the tidal air samples and forced expiration samples, furthermore, explains the difference which has been noted by others between the dead space calculated from the oxygen values and  $\text{CO}_2$  values. Theoretically, it can be shown that this dead space must be the same as long as the R.Q. of the expired and alveolar air are the same. The reason for the observed difference of a larger oxygen dead space is very probably that the alveolar samples were always obtained by the Haldane technique. Figure 4 shows the average expired air, E, and the alveolar air, A, values obtained in 27 experiments in which the alveolar  $\text{CO}_2$ ,  $\text{O}_2$  and R.Q. values were 38.1, 102 and .829 and the expired  $\text{CO}_2$ ,  $\text{O}_2$  and R.Q. were 27.8, 114 and .828 respectively. The Bohr formula,  $\frac{\text{deadspace}}{\text{tidal volume}}$

$= \frac{A-E}{A}$  can be represented graphically in figure 4 for the  $\text{O}_2$  as well as the  $\text{CO}_2$  values. The ratio  $\frac{A-E}{A}$  must be equal for both sides of the quadrangle as long as both A and E are on the same diagonal or R.Q. line.

If one assumes on the basis of the former experiments (6) that a Haldane sample had been delivered instead, the alveolar air values would have been located at point A-1 in figure 4. During the delivery of the sample, the concentration of point A would change and move up on the breath holding curve, B.H. (4).

The actual values cited above yield with the average tidal volume of 648 cc. (B.T.P.S.) an  $O_2$  and  $CO_2$  deadspace of 176 cc. If we subtract a 35 cc. apparatus deadspace from this figure, we obtain a personal deadspace (breathing through the nose) of 140 cc. Had Haldane samples been used instead, the personal deadspace

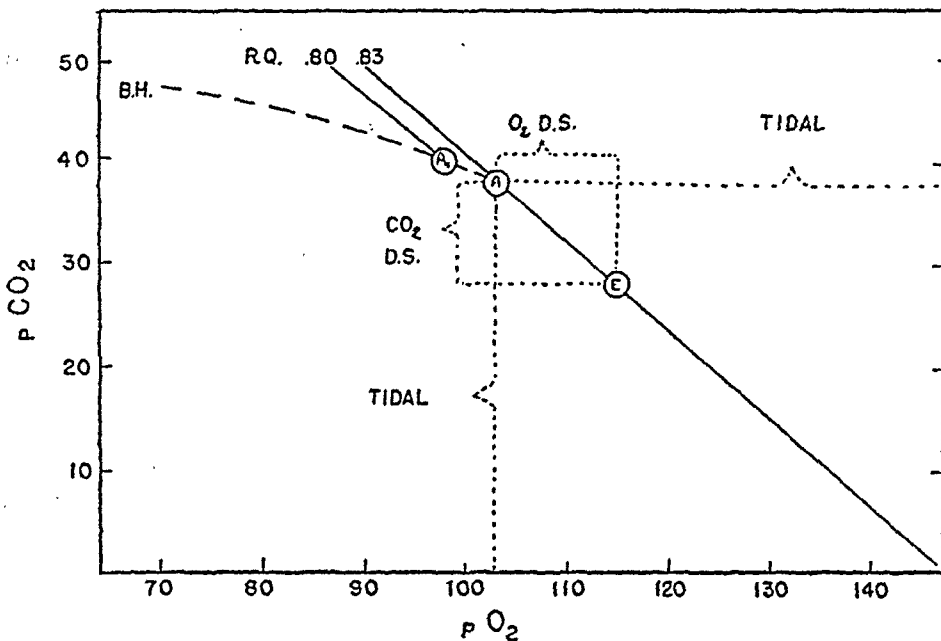


Fig. 4. A AND E REPRESENT the average alveolar and expired gas concentrations which fall upon the same R.Q. diagonal. The relative dead space to tidal volume ratio is shown graphically for  $O_2$  and  $CO_2$  and are the same as long as the R.Q. for E and A are the same. Point A-1 represents the alveolar value which would have resulted if the sample had been delivered from a forced expiration instead of from the end of a normal tidal expiration. The broken curve, B.H., describes the alveolar air changes when the breath is held at A (see text).

would have been 164 cc. for  $CO_2$  and 188 cc. for  $O_2$ . On the basis of the foregoing discussion it would appear that the deadspace values derived from Haldane samples are probably too large, particularly for the  $O_2$  deadspace.

*Effect of Unequal Ventilation and Bloodflow upon the Alveolar-Arterial Gradient.* In the foregoing discussion the concept of the theoretical mean alveolar gas concentration was based upon the assumption that all alveoli exchanged at the same R.Q. However, there is much evidence that ventilation is not equal in various parts of the lung (for a recent review see Rauwerda (9)) and Wearn *et al.* (10) have shown that the bloodflow is not constant in the alveoli. Thus, to appraise the variations in gas concentrations that may exist from one alveolus to the next and what effect this would produce upon the mean alveolar gas composition as well as upon the mean arterial gas tension, the variation of bloodflow and alveolar ventilation must be considered. Since both exert their effect upon the gas tensions, it is convenient to con-

sider them simultaneously and to evaluate the effect of the variation of alveolar ventilation to bloodflow ratio,  $V_a/F$  (11). From equation 3 we obtain by rearrangement:

$$\frac{V_a}{F} = \frac{.864 Q (A-V)O_2}{pC} \quad (4)$$

If we return to our original conditions of our example, figure 1, with a R.Q. equal to .8 then the mean alveolar  $pO_2$  and  $pCO_2$  must be 100 and 40 mm. Hg respectively, provided all the blood in all the alveoli is exchanging at this R.Q. Furthermore, these particular tensions are brought about by a  $V_a/F$  ratio of 1.0.

Theoretically other  $V_a/F$  ratios can occur ranging from 0 at the venous point to  $\infty$  at the inspired gas tension. Therefore, we may assume that the distribution of

TABLE 2

*A) Values which will be found at the various standards of deviation which are not apparent in figure 1*

	-3	-2	-1	MEAN	+1	+2	+3
$V_a/F$ .....	.45	.59	.77	1.00	1.30	1.60	2.20
R.Q.....	.53	.60	.68	.80	.98	1.17	1.38

*B) Blood and alveolar values which would be obtained if all alveoli exchanged at a  $V_a/F$  ratio of 1.0, (A), are compared with the mixture which would result if the  $V_a/F$  ratio varied around the mean (B) (see text)*

	$O_2$ CONTENT	$CO_2$ CONTENT	ALV. $pO_2$	ALV. $pCO_2$
A.....	19.65	48.00	100	40
B.....	19.55	47.90	99.4	39.7
Difference.....	0.10	0.10	0.6	0.3

the pulmonary bloodflow,  $F$ , as well as the ventilation,  $V_a$ , among the various alveoli is normal along a logarithmic scale.<sup>2</sup> The distribution of the ratio  $V_a/F$  will then also be normal along a logarithmic scale. Returning to our example, we can say that not all alveoli have a  $V_a/F$  of 1.0 but that some have a larger and others a smaller ratio distributed along a normal logarithmic distribution curve with the mean of the log 1.0. A standard deviation equal to the log 1.3 is arbitrarily chosen.

Table 2 A lists the various  $V_a/F$  ratios and R.Q.'s which then will be found at 1, 2, and 3 standard deviations from the mean  $V_a/F$  of 1.0 and figure 5 shows how this distribution affects the  $O_2$  and  $CO_2$  content of the arterial blood and the  $pO_2$  and  $pCO_2$  of the alveolar air. The resulting mixtures which would result for each of the 4 entities are calculated (the sum of the values of each class interval multiplied by the frequency of its occurrence) and listed in table 2 B to be compared with the theoretical mean value which would be obtained if no variation of  $V_a/F$  had oc-

<sup>2</sup> Among the various kinds of distribution curves, this is probably the simplest form which could be assumed for the distribution of a ratio.

curred as originally assumed. It can be seen that from a practical standpoint none of the resulting mixtures differ from the theoretical mean with the exception of the  $O_2$  content. Here we find that the arterialized blood leaving the lung capillaries has an  $O_2$  content 0.1 volume per cent lower than if all the exchange had gone on at a  $V_a/F$  of 1.0. The arterial blood leaving the capillaries is, therefore, represented by the open circle (fig. 5) and it can be seen that this is at an oxygen tension 8 mm. Hg lower than the mean alveolar  $O_2$  tension. (Although the  $CO_2$  content of the mixture also differs from the theoretical value by the same amount as the  $O_2$  content, it affects the arterial  $pCO_2$  by less than 0.2 mm. Hg.) It should be pointed out that the

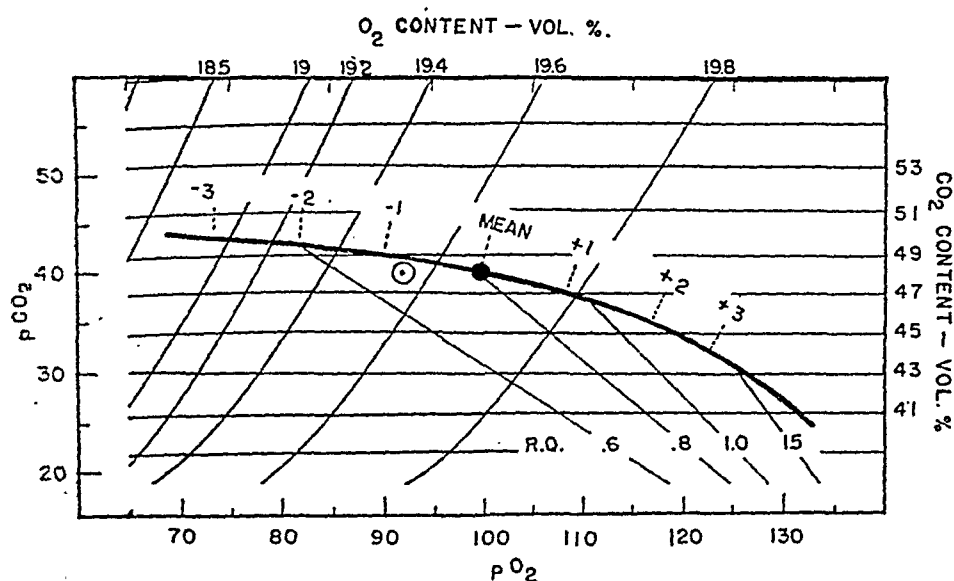


Fig. 5. DISTRIBUTION OF THE PULMONARY blood gas values and alveolar gas values when a normal logarithmic distribution of  $V_a/F$  is assumed around the mean value (solid circle). The resulting average mixture of the arterial blood will be altered only in respect to the oxygen content and arterial  $pO_2$  (open circle). The resulting average mixture of alveolar gases remains essentially unchanged. Thus, the distribution of  $V_a/F$  induces an alveolar-arterial  $pO_2$  gradient of 8 mm. Hg (see text).

mixed alveolar air as well as the mixed arterial blood points (table 2, B) no longer lie precisely on the blood R.Q. or gas R.Q. line of .8. This would suggest that the  $V_a/F$  ratios are not symmetrically, but unequally distributed in such a manner that the mixed values still maintain an R.Q. of .8. However, the above example is a first approximation and the discrepancy in this case may be considered negligible.

When, under otherwise identical conditions, various other venous points are chosen, the  $pO_2$  gradient is not appreciably altered. This means that a large or a small  $(A-V)O_2$  difference does not affect this gradient. On the other hand, if a larger deviation of  $V_a/F$  than log. 1.3 is assumed, the  $pO_2$  gradient can become appreciably larger. This may explain the greater alveolar-arterial oxygen gradient observed during exercise by Riley *et al.* (6). It should also be pointed out that an unequal distribution of  $V_a/F$  ratios will result in increased  $O_2$  gradients, particularly if a preponderance of alveoli have a  $V_a/F$  ratio which is smaller than the theoretical mean.

As discussed before, the standard deviation of this ratio was quite arbitrarily chosen and at present there is no way to ascertain the magnitude of this deviation nor whether the distribution of this  $V_a/F$  ratio is distributed symmetrically or asymmetrically. On the other hand, with the above assumption we can say that in the normal lung this deviation is no greater than the one we assumed because the gradient of 8 mm.  $pO_2$  obtained is of the same order of magnitude as that observed for the total gradient normally occurring (6). This gradient has so far been largely attributed to venous admixture arising from direct venous shunts, Thebesian and bronchial veins.

#### SUMMARY

An equation is derived which expresses the alveolar gas concentration in terms of the relative alveolar ventilation and pulmonary bloodflow. A graphical solution of this relationship is given which describes all the simultaneous  $O_2$  and  $CO_2$  concentrations which could theoretically exist for given mixed venous and inspired air tensions of these gases. Each of the possible alveolar air values, and therefore each  $R.Q.$ , is determined by a definite ventilation to bloodflow ratio. If the  $R.Q.$  is known, the theoretical mean alveolar air composition can be defined by this equation.

This theoretical concept of mean alveolar air presupposes that the ventilation to bloodflow ratio is the same in all the alveoli. Since this is unlikely, a variation of this ratio along a normal distribution curve is assumed. The mean alveolar air mixture resulting from such a distribution does not differ appreciably from the theoretical mean. The resulting arterial blood mixture, however, yields a lower  $pO_2$  tension, thus giving rise to an alveolar-arterial oxygen gradient.

Evidence is presented which indicates that the sampling of alveolar air from the last part of each normal expiration yields a value which is very close to the theoretical mean alveolar air composition, while the Haldane method of sampling yields  $CO_2$  values which are slightly too high and  $R.Q.$  values which are slightly too low. The latter method is responsible for the difference in dead space volumes when they are calculated separately from the  $O_2$  and  $CO_2$  values. Theoretically, the volumes should be the same if the expired air  $R.Q.$  and alveolar air  $R.Q.$  are equal.

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# ADRENERGIC MECHANISM OF VAGAL CARDIO-STIMULATION

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IN A previous paper (1) it was suggested that the presence of nervous structures in the mammalian heart which are capable of releasing an epinephrine-like substance under the action of acetylcholine, may provide an explanation for the findings of several authors, that stimulation of the vagus leads to acceleration of the atropinized heart of normal (2-6), sympathectomized (7, 8), or sympathectomized-adrenalectomized animals (9). In the present paper it is demonstrated that, in the isolated, perfused mammalian heart, stimulation of either the vagus or sympathetic nerves leads to the release of easily detectable amounts of a substance with epinephrine-like properties.

## METHODS

Cats, anesthetized with Dial (0.07-0.08 gm/kg. of body weight, intraperitoneally) were used. The heart was isolated *in situ* and perfused with Tyrode solution (at 38°C. and saturated with a mixture of 95% oxygen and 5% carbon dioxide), through a cannula introduced into the descending aorta at the level of the seventh rib. The preparation was so arranged that in addition to the coronary arteries, part of the mediastinal structures, including the stellate ganglia were also perfused. It has been observed in preliminary studies that under these conditions, the transmission of impulses through the stellate ganglion is maintained, as demonstrated by the fact that the cardiac response to the stimulation of the preganglionic sympathetic nerves persists unaltered for several hours.

The cervical vagal trunks were isolated and sectioned at the level of the larynx. They were excited a few millimeters below the point of section.

In 20 experiments, the cardiostimulating effect of the vagi was compared to that of preganglionic and postganglionic cardiac sympathetic fibers. To excite the preganglionic sympathetics, the thoracic sympathetic chains were isolated and cut 2 to 3 cm. from the stellate ganglion. The stimulating electrodes were placed a few millimeters caudally to the ganglion. To excite the postganglionic sympathetics, the stimulating electrodes were applied directly on the stellate ganglion. Modifications of the cardiac response to the excitation of the preganglionic sympathetics induced by nicotine, were used to evaluate the degree of synaptic block produced by this drug.

The nerves were excited with shielded, bipolar, silver electrodes. The excitation was performed with alternating current with a frequency of 50 c.p.s. The voltage

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was regulated by means of potentiometers. The nerves were excited with supra-maximal strength in all instances. The electrodes were kept separated from the neighboring tissues to avoid diffusion of the stimulating current. Atrial and ventricular contractions were recorded from the left auricle and apex, with isotonic levers. In some experiments cardiostimulation was induced by intracoronary injection of epinephrine or acetylcholine. These drugs were injected into the aortic cannula close to the heart and always in the same volume (0.1 c.c.).

In order to suppress the vagal cardiodepressor effect, i.e., to 'atropinize' the heart, Bellafoline (Sandoz)<sup>2</sup> was given in concentrations of 0.05 to 0.1 milligrams/liter Tyrode solution. Bellafoline was used because, like atropine sulphate, it abolishes the cardiodepressor effect of the vagus but without diminishing, as atropine usually does, the cardiostimulating effect.

Bitartrate of nicotine was used (1-2 mg/l. of Tyrode solution) to abolish transmission of impulses in the intracardiac ganglionic and stellate ganglion synapses.

The rectal cecum of the fowl was employed to detect the presence of an epinephrine-like substance in the cardiac perfusate. The technic was similar to the one described elsewhere (1), the only difference being that the perfusate was not collected in separate samples. Instead, it was allowed to drip directly upon the cecal preparation as it flowed from the heart. Since Bellafoline was present in the perfusate, it also served to suppress the cecal response to any acetylcholine liberated by the vagus during the experiments.

## RESULTS

### *Cardiostimulating Effect of the Vagus*

*Normal animals.* In all 99 experiments on normal animals, a cardiac response was regularly obtained by stimulating the vagi.

Before atropinization the stimulation of the vagus produced the well known depressor effect on the heart, a close relationship being observed between the strength of the stimulating current and the magnitude of the cardiac response. After the cardiodepressor effect of the vagus had been suppressed by atropinization (Bellafoline), the excitation of the vagus produced only stimulation of the heart, resembling the cardiac stimulation obtained by excitation of the cardiac sympathetic nerves.

The experiment shown in figure 1 is representative of these results. At 1, stimulation of the vagus induces a marked negative ino- and chronotropic effect on the heart. Once vagal stimulation is discontinued, the heart recovers from the depression very rapidly. Before its normal activity is restored, however, a short period of increased activity occurs during which the amplitude and frequency of the contractions are significantly augmented. At 2, the excitation of the left pre-ganglionic cardiac sympathetic nerve induces a marked increase in the amplitude and frequency of the heart's contractions. Between 2 and 3 the heart is atropinized by giving Bellafoline. Ten minutes later the vagus is again excited at 3. Now the heart reacts to the vagal excitation with a marked increase of the amplitude and

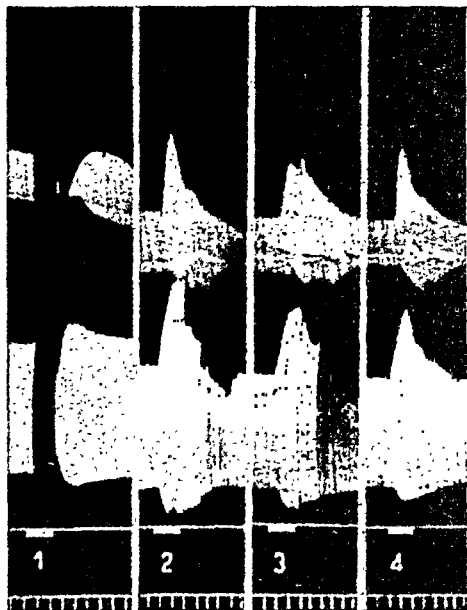
<sup>2</sup> We wish to thank the Oficina Cientifica Sandoz, Chile, for the generous supply of Bellafoline.

frequency of its contractions, an effect that very closely resembles the response of the heart to sympathetic stimulation as in 2 and 4.

Comparing the effects of vagal excitation in non-atropinized and atropinized hearts it is apparent that the cardiostimulating mechanism operates in both. In the non-atropinized heart, however, the cardiostimulating effect is masked by the predominant depressor influence. Upon suppression of the depressor effect by atropinization, the vagal cardiostimulating effect appears in its full strength.

The intensity of the cardiostimulating effect of the right as compared to the left vagus varied from animal to animal. Both vagi in any one animal usually had a similar cardiostimulating capacity. In some instances, however, either the left or the right vagus had a greater cardiostimulating effect.

Fig. 1. UPPER RECORD ATRIAL CONTRACTIONS; second record ventricular contractions; third record nerve excitation and injection marks; lowermost record time signal in 10 seconds. 1. Excitation of right vagus with 3 Volts. 2. Excitation of the right preganglionic sympathetic with 2 Volts. Between 2 and 3, Bella foline 0.2 mg/l. 3 and 4, respectively as in 1 and 2.



The vagal cardiostimulation consists primarily of an increased amplitude of contraction. There is usually, however, a more or less marked positive chronotropic effect. In the records shown, the chronotropic effect is not apparent because of the low speed of the kymographic recording.

*Cervical sympathectomized animals.* It was desired to exclude the possibility that the cardiostimulating effect of the vagus resulted from sympathetic cardio-stimulating fibers contributed to the vagus by the cervical sympathetic. For this purpose, 6 experiments were performed on cat hearts after the cervical sympathetic trunks, including the superior cervical ganglion, had been removed on both sides 15 to 30 days previously.

The results show that in the atropinized heart of these sympathectomized animals, the vagus regularly exerts a stimulating effect similar to that observed in the normal, atropinized heart.

#### *Influence of Nicotine*

In previous work (1), one of us has shown that the stimulating effect of acetylcholine on the isolated heart can be abolished by nicotine. It seemed interesting,

therefore, to study the influence of nicotine on the cardiostimulating effect of the vagus.

According to our results, nicotine, in concentrations adequate to block synaptic transmission in the stellate ganglion (abolition of the cardiostimulation produced by excitation of the preganglionic cardiac sympathetics), abolishes the cardiostimulating effect of the vagus.

The record in figure 2 corresponds to a typical experiment in the atropinized heart. At 1, it can be seen that excitation of the right vagus produces marked cardio-stimulation, particularly of the atria. At 2, during left preganglionic and at 3,



Fig. 2. UPPER TO LOWERMOST RECORDS, as in fig. 1. Atropinized heart. Bellafoline 0.1 mg/l. 1. Excitation of the right vagus with 3.5 Volts. 2. Excitation of the right preganglionic sympathetic with 4 Volts. 3. Excitation of the right postganglionic sympathetic with 3 Volts. 4. Nicotine (bitartrate), 2 mg/l. 5, 6 and 7, respectively as in 1, 2 and 3.

during left postganglionic sympathetic excitation, a similar cardiostimulating effect is obtained. At 4, nicotine is given. Under the action of this drug the heart shows an initial period of increased activity that passes off rather rapidly in spite of the fact that a constant nicotine concentration is maintained. Once this period of cardiostimulation has disappeared, the excitation of the vagus at 5 and of the preganglionic sympathetic nerve at 6 are completely ineffective. At 7, however, excitation of the postganglionic sympathetic exerts a cardiostimulating effect as intense as before nicotization.

#### *Release of an Epinephrine-like Substance*

Since it had been previously shown (1) that the cardiostimulation induced by acetylcholine in the isolated mammalian heart is produced by an epinephrine-like substance liberated in the heart itself, it seemed likely that the vagal cardiostimu-

lating effect might be attributed to the operation of a similar mechanism. To test the validity of this hypothesis, the presence of an epinephrine-like substance in the coronary perfusate during excitation of the vagus, was sought in 13 experiments.

The record in figure 3 shows a typical experiment of this series. At 1, vagal excitation exerts a strong stimulating effect on both the atria and the ventricles of the atropinized heart. At the same time, the rectal cecum of the fowl, which was continuously irrigated by the perfusate dripping from the heart, shows a marked diminution of its tonus. At 2, the excitation of the sympathetic exerts on both the heart and the intestine an action closely similar to that of the vagus. At 3, following

Fig. 3. ATROPINIZED HEART. Bellafolline 0.2 mg/l. Upper record cecal tonus; the rest of the records as in figs. 1. and 2. 1, Excitation of the right vagus with 3 Volts; 2, excitation of the right pre-ganglionic sympathetic with 3 Volts; 3, 0.1  $\mu$ g. of epinephrine; 4, 100  $\mu$ g. of acetylcholine.



the injection of 0.1 microgram of epinephrine there occurs a slight increase in the activity of the heart and a pronounced decrease of the intestinal tonus. At 4, following the administration of 100 micrograms of acetylcholine, cardiostimulation, especially of the ventricles, is seen. The rectal cecum, after a brief initial increment, responds with a marked diminution of its tonus. The initial increase in tonus is probably due to insufficient atropinization relative to the dose of acetylcholine used.

The decrease of the tonus of the rectal cecum under the action of the cardiac perfusate during the periods of vagal cardiostimulation, was observed without exception. Furthermore, the magnitude of the tonus decrease in any single preparation was closely proportional to the magnitude of the cardiostimulation. Usually, equivalent amounts of epinephrine-like substance were liberated by both vagi, but in some preparations either the right or the left vagal liberation predominated.

In a series of experiments it was shown that excitation of the cardiac nerves as well as the application of drugs modified the coronary flow, in addition to their action on cardiac activity. It seemed possible, therefore, that these changes in coronary flow could influence the tonus of the rectal cecum which was being directly irrigated by the perfusate flowing out from the heart. Control experiments showed, however, that similar changes of flow induced by modifying the perfusion pressure failed to alter the intestinal tonus to any significant extent.

#### DISCUSSION

The cardiostimulating effect of the vagus observed in our experiments is apparently caused by the liberation in the heart of a substance which, like epinephrine, induces relaxation of the rectal cecum of the fowl. The active substances recovered in the perfusate from the atropinized heart under the action of the cardiac sympathetic nerves or acetylcholine, also induce a decrease of the cecal tonus. The epinephrine-like nature of the substance (sympathin) released in the mammalian heart by the sympathetic nerves has been repeatedly demonstrated (10-13). The epinephrine-like properties of the active substance released in the heart by acetylcholine, have been demonstrated by Hoffmann *et al.*, on a variety of biological tests (1).

On the basis of these facts, the substance liberated by the vagus in the heart appears most likely to be epinephrine-like in nature.

The abolition by nicotine of the vagal cardiostimulating effect strongly supports the hypothesis that the elements responsible for the liberation of this epinephrine-like substance are intracardiac ganglionic neurones. The adrenergic intracardiac ganglia are presumably normally controlled by the vagus, i.e., presumably make synaptic connections with preganglionic vagal cardiac fibers. This assumption is supported: a) by the fact that they are activated by excitation of the vagus—possibility of being indirectly stimulated by acetylcholine released by the vagus in the heart and reaching them via the blood vessels seems remote; and b) by morphological evidence (14), according to which it is usually accepted that the intracardiac ganglia belong, without exception, to the vagal system.

These facts provide additional support for the hypothesis advanced in previous work (1), that acetylcholine stimulates the heart by activating adrenergic intracardiac structures.

The presence in the heart of adrenergic ganglia connected with the vagus, would represent an exception in the classical scheme of peripheral integration of the autonomic nervous system, according to which the parasympathetic postganglionic neurones are universally cholinergic in nature. Another exception is found in the sweat glands in which, as shown by Dale (15), the sympathetics synapse with cholinergic post-ganglionic neurones. How far the existence of adrenergic ganglia is restricted to the heart or applies to other parasympathetic innervated structures, cannot be ascertained on the basis of evidence available at present. It is interesting to recall, however, Koppányi's (16) demonstration that in atropinized animals, acetylcholine produces relaxation of the intestine and this inhibiting effect of acetylcholine can be abolished by nicotine.

Our experiments provide more direct evidence in support of Haney's (9) assumption that adrenergic ganglia exist in the heart which are responsible for the acceleration induced by acetylcholine in the denervated heart of atropinized dogs.

The fact that the cervical vagus retains its cardiostimulating capacity in chronic cervically sympathectomized animals, excludes the possibility that the vagal cardiostimulating effect could be due to sympathetic fibers in the vagus.

#### SUMMARY

In the atropinized, isolated cat heart, perfused with Tyrode solution, the excitation of the cervical vagus induces positive ino- and chronotropic effects on both atria and ventricles. This vagal cardiostimulating effect is abolished by nicotine in concentrations of 1 to 2 mg/l. Along with the cardiostimulation produced by the vagus, an epinephrine-like substance is released in the heart, the perfusate acquiring the property of relaxing the rectal cecum of the fowl.

A similar effect on cardiac activity and the release of a substance that exerts a similar relaxing effect on the cecal tonus is obtained by excitation of the pre- or post-ganglionic cardiac sympathetic and by intracoronary injection of acetylcholine.

These results are interpreted as indicative of the existence in the heart of adrenergic ganglia connected with the vagus.

We wish to thank Prof. Harold C. Wiggers for the careful revision and most valuable critical survey of the manuscript.

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# A STUDY OF THE INFLUENCE OF DL-TRYPTOPHAN UPON BLOOD SUGAR LEVELS<sup>1</sup>

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**T**URNER and Crowell (1) published observations which seem to indicate that daily oral administration of 0.14 to 0.32 gm. of DL-tryptophan to normal adults produced symptoms of mild hypoglycemia and an associated reduction of the blood serum glucose level. Howard and Modlinger, in a recent report (2), indicate that their attempts to confirm these findings were unsuccessful. The latter authors were unable to demonstrate any consistent lowering of the blood sugar by administration of tryptophan orally to adults.

An implication of tryptophan in the regulation of the serum glucose suggests a possible involvement of the pyridine nucleotides which are known to be concerned in the oxidation of carbohydrates *in vivo*. The recent work (3) elucidating the mechanism of pyridine nucleotide synthesis in the presence of pyridoxine and tryptophan suggests a possible mechanism through which added tryptophan might influence carbohydrate metabolism. Data indicating that nicotinamide administered parenterally led to significant reduction of the fasting blood glucose levels and insulin requirements of diabetic humans have been reported by Neuwahl (4).

The following experiments were designed to study the influence of tryptophan on the blood sugar level in normal rabbits and man.

## EXPERIMENTAL

*Experiment 1.* Five adult rabbits which had been maintained 14 months in the laboratory on a commercial rabbit pellet diet<sup>2</sup> without subsidiary fresh greens were fasted from 5:00 P.M. to 9:00 A.M. (16 hours) on successive days. Each morning the animals were placed in an animal box and 20 cm. of blood was obtained by ear puncture for duplicate determinations of the blood sugar level by the method of Reinecke (5). After a preliminary control period of 5 days, the animals were given 25 mg/kg. body weight of DL-tryptophan orally. The tryptophan was administered as a 0.5 per cent solution in distilled water. The appropriate volume of tryptophan solution was added to the water cup and the volume made up to 50 ml. with tap water. This was given at 5:00 P.M. at the beginning of the fast. It was found that the animals would regularly consume this amount of liquid during the 16-hour fast. Blood sugar determinations were done each morning at the end

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<sup>2</sup> Purina pellets.

of this 16-hour fast. After 7 days of tryptophan administration, the animals were given only tap water and the fasting blood sugar levels determined at intervals for 10 weeks. Food consumption was measured before and during tryptophan administration and was found to be substantially unchanged. The animals were weighed daily and the body weights found to be constant throughout.

Table 1 indicates the data obtained. The daily mean of the 5 animals is recorded for the sake of brevity. The arithmetic mean of the 24 blood sugar determinations before tryptophan was found to be 117.2 mg. per cent with a standard deviation of 7.5. The arithmetic mean of the 20 determinations during tryptophan feeding was 99.1 mg. per cent with a standard deviation of 7.3. The difference of the two

TABLE 1. DAILY BLOOD SUGAR DETERMINATIONS IN FIVE RABBITS BEFORE, DURING AND AFTER ORAL TRYPTOPHAN ADMINISTRATION<sup>1</sup>

BEFORE TRYPTOPHAN			DURING PERIOD OF TRYPTOPHAN ADM.			AFTER TRYPTOPHAN DISCONTINUED		
Day	Mean	S.D.	Day	Mean	S.D.	Day	Mean	S.D.
1	113	7.8	1	100	5.9	1	98	3.1
2	120	6.4	2	99	10.2	3	90	7.7
3	118 <sup>2</sup>	3.9	3	100	6.8	4	90	6.2
4	112	6.5	4	98	5.2	5	89	6.4
5	123	6.1				6	98	3.1
						7	95	6.6
						19	92	3.6
						35	114	17.0
						59	117 <sup>2</sup>	4.4
						61	100 <sup>2</sup>	7.5
						62	98 <sup>2</sup>	4.5

<sup>1</sup> The daily mean blood sugar is listed for the sake of brevity. In statistical analyses mentioned in the text, the arithmetic mean of individual values was used, however.

<sup>2</sup> Mean determined from 4 values instead of 5.

means before and after tryptophan administration is therefore 18.1 mg. per cent. The standard error of the difference of the means was found to be 3.54.<sup>3</sup> The critical ratio, determined by dividing the difference of the means (18.1) by the standard error of the difference of the means (3.54), was found to be 5.1, which is considered to be of statistical significance. (Critical ratios greater than 2.5 are usually regarded as significant)(6.) The fact that this statistically significant fall in blood sugar occurred at the time of tryptophan administration suggested a specific effect of tryptophan.

<sup>3</sup> The standard error of the difference between the means was calculated by the following formula: Standard Error = Square Root of  $\left(\frac{(S.D.)^2}{n_1} + \frac{(S.D.)^2}{n_2}\right)$  in which 'S.D.' represents the standard deviation of the entire population (combined groups of pre- and post-tryptophan values), 'n<sub>1</sub>' represents the total number of determinations before tryptophan, and 'n<sub>2</sub>' represents the total number of determinations after tryptophan. The standard deviation (S.D.) was calculated from the following formula: S.D. of total population = Square Root of  $\left(\frac{\text{Sum of squares of all ind. values}}{n} - \text{Mean}^2\right)$  in which 'n' represents the total number of determinations before and after tryptophan and the 'Mean' represents the arithmetic mean of all blood sugar determinations before and after tryptophan.

However, it will be noted (table 1) that the blood sugar did not return to the original levels after tryptophan was discontinued. This suggested either that by coincidence the animals became accustomed to the procedure at the time of the tryptophan treatment, or that tryptophan did cause a reduction in fasting blood sugar which appeared immediately and has persisted for many weeks.

*Experiment 2.* Approximately 3½ months later, the preceding experiment was repeated with the same animals, with the following differences in procedure: a smaller tryptophan dosage and another blood sugar method (measuring 'true blood sugar')

TABLE 2. DAILY FASTING BLOOD SUGARS IN FIVE RABBITS BEFORE AND AFTER ORAL TRYPTOPHAN ADMINISTRATION<sup>1</sup>

BEFORE TRYPTOPHAN				AFTER TRYPTOPHAN			
Day	No. of det.	Mean	S.D.	Day	No. of det.	Mean	S.D.
1	4	97.6	8.5	1	5	101.0	5.0
2	5	88.3	10.1	2	5	85.3	7.9
3	5	103.0	9.4	3	5	89.7	7.2
4	5	98.9	10.9	4	5	105.0	2.2
5	4	112.0	7.0	5	5	85.1	13.7
6	5	101.0	13.7	6	5	95.2	8.2
7	5	103.0	10.1	7	5	91.7	7.6
8	5	104.0	4.4	8	5	88.2	7.5
9	5	97.9	6.8	9	5	91.5	6.0
10	5	94.6	15.7	10	5	88.1	5.4
11	5	90.1	10.6	11	5	85.4	7.2
12	5	90.1	15.6	12	5	92.4	4.1
13	5	96.4	8.1	13	5	92.8	3.8
14	4	101.0	15.2				
15	5	95.5	11.3				
16	5	111.0	8.1				
17	5	102.0	7.9				

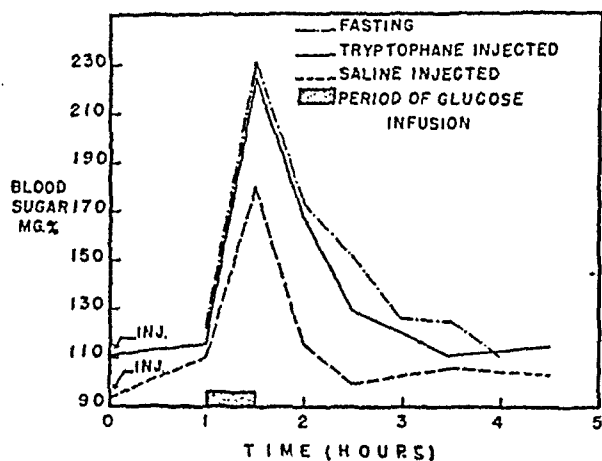
<sup>1</sup> The daily mean of fasting blood sugar levels of either 4 or 5 animals is indicated in the table, together with its standard deviation. Similarly the daily mean fasting blood sugar level of the same 5 animals after tryptophan is listed. In calculating the critical ratio cited in the text, however, the arithmetic mean of individual animal values was employed rather than the mean values listed above.

were used and a greater number of observations before and after tryptophan administration was obtained. The details follow: five adult rabbits maintained on a rabbit pellet diet and tap water were placed in individual cages. Following repeated 16-hour fasts (5:00 P.M. to 9:00 A.M.) during which only tap water was made available, blood sugars were determined each morning on 17 successive occasions, by Nelson's micro-method (7) using the Coleman Universal spectrophotometer. The DL-tryptophan in a concentration of 0.1 per cent in distilled water was then placed in the troughs in each cage, every evening at 5:00 P.M., in a dose of 10 mg/kg. body weight. This total quantity of fluid was sufficiently small (35-45 ml.) that the entire amount would be consumed by the end of the 16-hour period, food being withheld as before. Blood sugars were again determined each morning at the end of the 16-hour fast.

The results are summarized in table 2. The mean value of the 82 determinations prior to tryptophan administration was found to be 99.0 mg. per cent with a standard deviation of 12.4. The mean value of the 65 determinations following the administration of oral tryptophan was found to be 91.4 mg. per cent with a standard deviation of 9.88. The difference between the two means before and after tryptophan administration is 7.6 mg. per cent. The standard error of the difference of the means was found to be 1.99. The critical ratio was found to be 3.8, which is considered to be of statistical significance. This appears to confirm the results obtained in *experiment 1*, although the magnitude of the fall in blood sugar after tryptophan is not as great.

*Experiment 3.* Two adult female rabbits were used to study the effect of tryptophan on the glucose tolerance test. The animals were fasted 16 hours, placed in rabbit boxes and a preliminary blood sample obtained. They were then given 0.5 gm/kg. of glucose as a 10 per cent pyrogen-free solution in distilled water over

Fig. 1. GLUCOSE TOLERANCE TESTS in the fasting state, and following tryptophan and saline administration, in rabbits. The mean values of 2 experiments, 2 days apart, are plotted.



a 30-minute period. Blood samples were obtained at the close of the infusion and at 30, 60, 90, 120, 150, 180, and 210 minutes thereafter. The Reinecke glucose method was used. The animals were maintained on a commercial rabbit food for one week prior to the testing. Four control tests on 2 rabbits indicate good reproducibility. The same animals were then used for glucose tolerance tests after the injection of 25 mg/kg. body weight of DL-tryptophan in a 0.5 per cent solution intraperitoneally in *rabbit 6* and an equal volume of solution per kilogram of normal saline in *rabbit 7* immediately before the tolerance test. This procedure was repeated 2 days later on the same animals.

Figure 1 illustrates the glucose tolerance tests in the fasting state prior to, and following administration of saline and tryptophan. Although the saline injected animals exhibited a significantly lower glycemia, there is no apparent effect exerted by tryptophan injection.

*Experiment 4.* The 2 adult female rabbits, 6 and 7, used in *experiment 3* were supplemented with *rabbit 8* of similar size. Food was removed from the cages at 10:00 A.M. Four hours later blood was taken for blood sugar determinations and the animals were injected intravenously, through an ear vein as follows. *Rabbit 6*

received 25 mg/kg. of nicotinamide solution in sterile distilled water; rabbit 7 received an equivalent amount of normal saline on a body weight basis; and rabbit 8 (which had received no previous tryptophan) received 25 mg/kg. of DL-tryptophan as a 0.5 per cent solution in sterile distilled water. Pyrogen-free water and saline were used. Blood was drawn at hourly intervals for 3 hours thereafter for glucose determinations (Reinecke). Two days later the same experiment was repeated after a 16-hour fast.

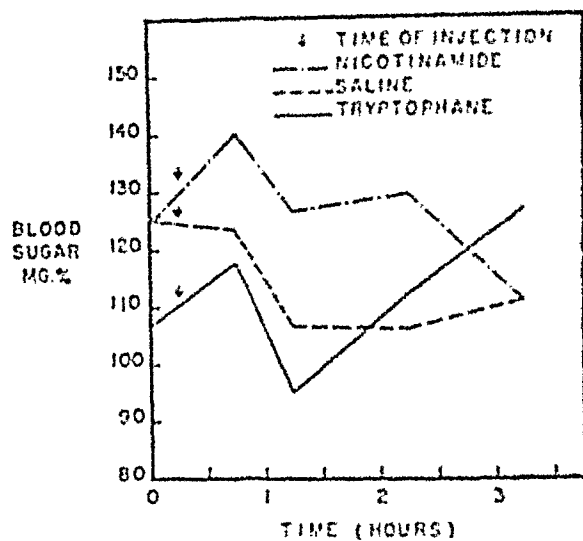


Fig. 2. Effect of injections of nicotinamide, tryptophan, and saline on blood sugar levels in rabbits. Each point represents the mean value of 2 experiments, 2 days apart.

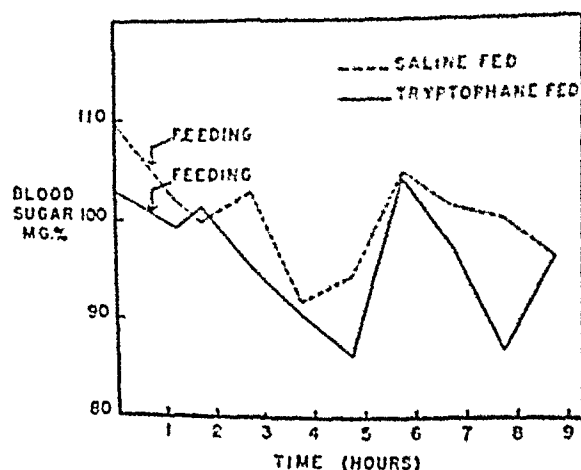


Fig. 3. Hourly blood sugar determinations over an 8-hour period following tube feeding of tryptophan and saline. Each point represents the mean blood sugar of 2 rabbits.

The mean value of the determinations is plotted in figure 2, and inspection reveals no consistent effect of either nicotinamide or tryptophan on the blood sugar level.

*Experiment 5.* Four young rabbits that had never received tryptophan previously were deprived of water and food from 5:00 P.M. until the completion of the experiment 24 hours later. At 8:30 A.M., 15½ hours after the removal of food and water, 2 of the animals were given 10 mg/kg. of tryptophan in 0.1 per cent aqueous solution per stomach tube, and the other 2 were given isotonic saline by

the same route. Blood for sugar determinations (method of Nelson, 7) was then drawn at  $\frac{1}{2}$ , 1, 2, 3, 4, 5, 6, 7, and 8 hours after the tube feeding. The results are plotted in figure 3. Examination of these data reveals no significant difference between the blood sugar levels of the tryptophan-fed animals and the control animals.

*Experiment 6.* This experiment was designed to supplement the preceding one. Whereas in *experiment 5* the blood sugars of only the first 8 hours following tryptophan feeding were determined, in the present experiment the blood sugars from the 7th to the 16th hour following tryptophan feeding were obtained. Four

Fig. 4. HOURLY BLOOD SUGAR DETERMINATIONS in rabbits from the 7th to the 16th hour following tube feeding of tryptophan and saline. Each point represents the mean blood sugar of 2 animals.

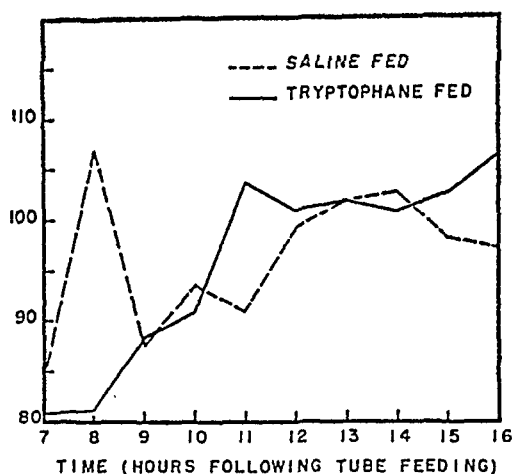


TABLE 3. DAILY MEAN OF BLOOD SUGAR LEVELS OF FOUR ADULT HUMANS BEFORE AND AFTER ORAL TRYPTOPHAN ADMINISTRATION<sup>1</sup>

BEFORE TRYPTOPHAN			AFTER TRYPTOPHAN		
Day	Mean	S.D.	Day	Mean	S.D.
1	95.8	10.2	1	92.8	5.1
2	85.3	20.3	2	83.3	8.6
3	86.5	8.5	3	82.0	9.0
4	94.5	4.5	4	89.3 <sup>2</sup>	6.5

<sup>1</sup> Statistical data cited in the text are based on individual values rather than these daily mean values.

<sup>2</sup> Mean determined from 3 values instead of 4.

rabbits, none of whom had previously been fed tryptophan, were weighed, and then deprived of food and water. Sixteen hours later 10 mg/kg. body weight of tryptophan in 0.1 per cent solution was given per stomach tube to 2 of the animals, and an equal volume of isotonic saline per kilogram body weight was given to the other 2 animals. Blood sugars were determined (method of Nelson, 7) hourly from the 7th to the 16th hour following this feeding, food and water being withheld until the conclusion of the experiment. The results plotted in figure 4 indicate no significant effect of tryptophan on the blood sugar.

*Experiment 7.* Four young adult human volunteers were asked to appear in the laboratory each morning for fasting blood sugar determinations (method of Reinecke, 5) having taken no food over the previous 14 hours. After a pre-

liminary period of 4 days, the subjects were given weighed portions of DL-tryptophan in a dosage of 25 mg/kg. body weight, which they swallowed with a little water at 8:00 A.M. At 10:00 A.M. they appeared as usual for blood sugar determinations. Table 3 indicates the daily mean values obtained. The mean blood sugar before tryptophan administration, based on the arithmetic average of all individual values, was 90.8, whereas the mean blood sugar after tryptophan was 84.7, with a mean drop of 4.2. Calculation of the standard error of the means gives a value of 4.02. The critical ratio therefore is 1.04, which is not significant statistically. The subjects did not experience unusual symptoms. This experiment, therefore, does not reveal any significant effect of tryptophan on the blood sugar.

#### DISCUSSION

In a series of 7 experiments, 6 conducted on rabbits and one on human subjects, it has not been possible to demonstrate any consistently hypoglycemic action of tryptophan. Although in the first 2 experiments in which the amino acid was fed by mouth to rabbits, a small drop in the mean blood sugar was demonstrated following tryptophan administration, this was not reproduced when tryptophan was given either by stomach tube or intravenously. The employment of a blood sugar method (5) which measures total reducing substances in *experiment 1* may possibly explain the greater drop in blood sugar obtained in this experiment than in *experiment 2*, in which a method (7) measuring the true blood sugar was employed. Turner and Crowell (1) used the Folin-Wu blood sugar method, which also measures total reducing substances, and it is conceivable that the results they obtained may in part have resulted from a fall in these non-glucose substances. *Experiment 7* in which 4 young adult human subjects participated also failed to demonstrate any hypoglycemic effect following tryptophan administration.

#### SUMMARY

The results of 7 experiments are reported designed to determine the effect of tryptophan administration on the fasting blood sugar levels, when administered orally, intravenously, or by stomach tube, and on the intravenous glucose tolerance test, when administered intraperitoneally. The studies were made with rabbits and man and no consistently hypoglycemic effect was observed following administration of tryptophan.

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# DISCHARGE OF ADRENOCORTICOTROPHIC HORMONE IN THE ABSENCE OF NEURAL CONNECTIONS BE- TWEEN THE PITUITARY AND HYPOTHALAMUS<sup>1</sup>

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**C**HANGES in the weight, histology and chemistry of the adrenal cortex serve as convenient and specific indices ('target gland indices') to measure the rate of discharge of adrenocorticotrophic hormone (ACTH) from the adeno-hypophysis under various experimental conditions (1). The demonstration by Uotila (2) and Brolin (3) that pituitary stalk section has no effect upon the increase in adrenal weight which follows chronic exposure of rats to a cold environment suggests that neural connections between the hypothalamus and pituitary are not necessary for the pituitary-induced adrenal hypertrophy associated with chronic stress. Inasmuch as ACTH is discharged from the adeno-hypophysis within a few minutes after the onset of stress, i.e., long before changes in adrenal weight become measurable (1), it was deemed important to determine whether the immediate discharge of ACTH is regulated by a hypothalamic-hypophyseal neural mechanism.

## METHODS

Male rats from the Sprague-Dawley farm were divided into 3 groups. One group of 9 rats remained as controls; a second group of 12 was completely hypophysectomized; and in the third group of 19 rats, the pituitary stalks were sectioned satisfactorily.

Complete hypophysectomy was performed in the routine manner. Pituitary stalk section was accomplished by a modification of the routine procedure for hypophysectomy. The drill hole between the sphenoid and occipital bones was extended laterally. The membrane of the sella was opened and the isthmus of the adeno-hypophysis was split with a dental probe just ventral to the posterior lobe. The lateral lobes of the anterior pituitary were spread apart with a retractor. A fine-tipped pipette was then introduced into the pituitary fossa between the blades of the retractor and vigorous suction applied to the roof of the sella turcica. The posterior pituitary was sucked out, and in practically all rats a variable fraction of the anterior pituitary was also withdrawn. All animals were discarded in whom

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it was obvious at the time of operation that more than one-half of the anterior pituitary tissue was removed. Nineteen stalk-sectioned animals were deemed satisfactory for purposes of the experiment; but at autopsy, 7 of these 19 rats were found to have a quantity of anterior pituitary tissue less than one-quarter of normal. Data from these 7 animals are dealt with separately.

Three separate criteria were employed to determine the completeness of the severance of neural connections between the pituitary and hypothalamus, as follows: gross inspection at autopsy, water metabolism and histological examination of the supraoptic nuclei. Gross inspection at autopsy showed that none of the stalk-sectioned animals had any tissue connecting the base of the brain and the pituitary. The daily water intake and urine output of 10 of the 12 stalk-sectioned animals were measured; 9 of the 10 rats exhibited marked polyuria and polydipsia (+ in table 1) and the remaining rat showed a mild diabetes insipidus (+ - in table 1). The supraoptic nucleus was examined histologically in 7 stalk-sectioned animals. Serial sections of the nucleus were stained with toluidine blue. From 5 to 48 days elapsed between stalk section and autopsy, immediately prior to which the rate of ACTH discharge in response to acute stress was measured. Five days after stalk section the supraoptic nucleus was normal for the most part. However, there were scattered areas in which cells showed some irregularity of outline, dense peripheral Nissl substance and eccentric karyolytic nuclei. After 23 days, definite signs of cellular degeneration were apparent; cells were observed in all stages of degeneration and there was a moderate degree of gliosis. Although no cell counts were made, it was clearly evident that a decrease in the number of cells had occurred. After 48 days, more than one-half of the cells of the supraoptic nucleus had disappeared. Harris (4) has reviewed the experimental studies covering a number of species which demonstrate that section of the infundibulum is followed by degenerative changes in the supraoptic nucleus.

Integrity of function of the adeno-hypophysis was evaluated by measuring body, adrenal and testicular weights. Ability of the pituitary to discharge ACTH was determined by the degree of depletion of adrenal ascorbic acid in response to the administration of histamine. The ascorbic acid content of the adrenals is under the specific regulatory control of the pituitary and can be employed as a measure of ACTH discharge (1). The degree of depletion of adrenal ascorbic acid in response to histamine was measured as follows: The rat was anesthetized with sodium pentobarbital and the left adrenal removed as a control for the analysis of ascorbic acid. Histamine acid phosphate, 1.0 mg/100 gm. body weight, was injected slowly via a tail vein, over a period of about 3 minutes. One hour later the right adrenal was removed for analysis. The adrenals were dissected free of fat, weighed to the nearest 0.05 mg. on a torsion balance and then dropped into a centrifuge tube containing a few grains of sand and 12.0 ml. of 4.0 per cent trichloroacetic acid. A stirring rod, flattened at the tip, was used to crush and grind the adrenal tissue against the sand at the bottom of the tube. Two 4.0 ml. aliquots of the extract were analyzed for total (reduced plus dehydro) ascorbic acid by the method of Roe and Kuether (5). The degree of depletion of adrenal ascorbic acid,

TABLE 1. DISCHARGE OF ACTH FROM THE ADENOHYPHYPHYSIS OF STALK-SECTIONED RATS IN RESPONSE TO HISTAMINE STRESS

GROUP	BODY WEIGHT		BOTH ADRENALS WT., MG.	TESTES WT., GM.	REDUCTION IN ADRENAL ASCORBIC ACID, MG/100 GM.	POLYURIA AND POLYDIPSIA	DEGENERATION OF SUPRAOPTIC NUCLEUS
	Initial	Final					
Control	75	270 (46) <sup>1</sup>	28.3		158		
	60	264 (46)	24.4		201		
	62	276 (46)	34.8		199		
	192	250 (13)	27.6	2.77	208		
	194	240 (13)	27.6	2.71	160		
	230	298 (14)	32.0	2.60	199		
	200	264 (14)	29.9	2.90	218		
	190	270 (15)	32.5	3.00	205		
	170	210 (15)	26.9	2.16	250		
Average.....					200 ± 9		
Hypophysectomized	53	72 (51) <sup>2</sup>	6.4		-21 <sup>3</sup>		
	105	100 (51)	8.2		5		
	160	154 (13)	11.8	0.89	-26		
	150	140 (13)	17.7	0.22	35		
	172	170 (14)	12.8	0.70	-1		
	164	145 (14)	18.1	0.56	6		
	152	140 (15)	13.8	0.16	-10		
	156	140 (15)	15.4	0.71	2		
	270	250 (20)	14.3	1.29	13		
	274	220 (20)	15.5	1.00	46		
	268	233 (20)	14.4	1.15	17		
	270	244 (20)	14.6	1.01	18		
Average.....					7 ± 6		
Stalk-sectioned	170	156 (5) <sup>2</sup>	24.4		203		o+ <sup>4</sup>
	168	136 (5)	29.5		190		
	200	240 (23)	29.6	2.75	258	+	++
	200	200 (23)	25.7	2.70	303	+	++
	218	250 (23)	28.6	3.15	343	+-	
	258	250 (48)	29.1	2.98	185	+	+++
	260	245 (48)	36.4	3.60	231	+	++++
	278	210 (48)	29.7	2.93	123	+	
	226	255 (32)	31.6	3.22	288	+	
	264	292 (32)	36.8	3.99	257	+	++
	254	264 (32)	36.9	2.23	319	+	
	258	288 (32)	37.2	3.60	210	+	++
Average.....					242 ± 19		

<sup>1</sup> Figures in parentheses denote number of days elapsed between measurement of initial and final body weights.

<sup>2</sup> Figures in parentheses denote number of days elapsed between operation and autopsy.

<sup>3</sup> A minus sign indicates that the right adrenal had a greater concentration than the left.

<sup>4</sup> The symbols have the following meanings: o+ scattered areas of chromatolysis, no cell loss; ++ cells in all stages of degeneration, moderate cell loss; ++++ loss of more than 50 per cent of cells, all stages of degeneration and gliosis.

as measured by the difference in concentration between the left and the right gland, is proportional to the amount of ACTH discharged from the adenohypophysis.

### RESULTS

Section of the infundibulum interfered with growth. Of the 12 stalk-sectioned rats included in table 1, 6 gained slightly in weight and 5 actually lost weight. All the completely hypophysectomized rats lost weight, except one animal which was operated upon at a very young age. The testes and adrenals of the stalk-sectioned group were equal in weight to those of the control series; in contrast, the gonads and adrenals of the hypophysectomized rats were markedly atrophic. It appears that gonadotrophins and ACTH were released from the stalk-sectioned pituitaries in quantities sufficient to maintain the weights of the testes and adrenals, respectively. On the other hand, it is possible that inadequate quantities of growth hormone were being discharged from the adenohypophysis of the stalk-sectioned rats. However, growth is such a complex phenomenon that a deficiency of pituitary growth hormone may be only one of several possible factors responsible for the inadequate gain in weight of the stalk-sectioned animals. Indirect evidence that the thyrotrophic function of the anterior pituitary was not disturbed exists in the fact that the diabetes insipidus occasioned by stalk section persisted for the duration of the period of observation.

None of the completely hypophysectomized rats responded to histamine with a significant reduction in adrenal ascorbic acid (table 1). In contrast, the reduction in adrenal ascorbic acid in the stalk-sectioned rats given histamine was as marked as that in the control group similarly treated.

Of a total of 19 stalk-sectioned animals, 7 were found at autopsy to have either no detectable pituitary tissue or an amount which was less than one-quarter the normal mass of this gland. These 7 rats have not been included in table 1. Their adrenals and gonads were atrophied and the adrenal ascorbic acid response to histamine was either completely negative or intermediate between that of the controls and that of the completely hypophysectomized animals.

### DISCUSSION

The maneuvers of vigorous suction applied to the roof of the sella and of separation of the lateral lobes of the adenohypophysis are reasonable assurances in themselves that neural connections between the pituitary and the hypothalamus were completely disrupted. At autopsy the brain was very carefully lifted from the base of the skull in order to reveal any possible residual connections between the hypophysis and the hypothalamus. In control animals the stalk was readily seen, but in none of the stalk-sectioned animals could any infundibular tissue be observed. The degenerative changes in the supraoptic nucleus provide further evidence that the stalk had been sectioned. Finally, the polyuria and polydipsia which developed in the stalk-sectioned animals adds additional support of a physiological nature to the anatomical evidence that the infundibulum had been sectioned. Thus it is reasonably certain that the stalk-sectioned animals in this study lacked

neural connections between the adenohypophysis and the hypothalamus. However, it is possible that a 'hypophyseal portal system' regenerated and reestablished vascular connections between the pituitary and the hypothalamus.

Uotila (2) investigated the response of the adrenals of stalk-sectioned and normal rats to chronic exposure to cold, and concluded that the absence of the infundibulum did not prevent the adrenal hypertrophy which normally occurs from such exposure. Brodin (3) conducted similar studies and his results agree with those of Uotila. It thus appears that neural connections between the pituitary and the hypothalamus are not essential for the increased pituitary adrenocorticotrophic activity which results from the continuous application of stress. Furthermore, the present study demonstrates that the prompt discharge of ACTH which occurs within an hour after the application of a noxious agent is also independent of neural connections between the hypothalamus and the adenohypophysis.

The data of this report would appear to indicate that the rate of discharge of ACTH in stalk-sectioned rats, as judged by the depletion of adrenal ascorbic acid, is equal to that in intact rats. However, the possibility remains that stalk-section may modify the response to acute stress. The histamine stress employed in these studies caused a nearly maximal reduction in adrenal ascorbic acid. In order to detect small quantitative differences between control and stalk-sectioned rats in the rate of discharge of ACTH, a dose of histamine should be employed which produces less than the maximal reduction in adrenal ascorbic acid. When one considers the fact that only one-half of the normal mass of pituitary tissue was found at autopsy in most of the stalk-sectioned animals and that the blood supply of the gland was probably interfered with by the surgical procedure, it is highly significant that the reduction of adrenal ascorbic acid in response to histamine was as great in the stalk-sectioned as that in the intact control rats.

Harris (4) has stated that, "...nervous stimuli might cause the liberation of some substance into the capillary sinusoids of the median eminence, this substance then being transported via the hypophyseal portal vessels to excite or inhibit the pars distalis." There appears to be good evidence in the rabbit that neurovascular transmission from the hypothalamus to the adenohypophysis is the mechanism regulating the release of a pituitary gonadotrophin responsible for ovulation. Since regeneration of the hypophyseal portal vessels occurs after division of the stalk in rats (4), the results of the present study do not rule out the possibility that a neuro-humor, arising in the hypothalamus and passing to the adenohypophysis via a hypophyseal portal system, mediates the release of ACTH in response to the application of stress.

#### SUMMARY

In rats with pituitary stalk section the adrenal cortical response to an acute stress is similar to that of control animals. It is therefore concluded that the prompt release of adrenocorticotrophic hormone from the adenohypophysis in response to stress is not dependent upon neural connections between the hypothalamus and the anterior pituitary.

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# RENOTROPHIC-ANDROGENIC AND SOMATOTROPHIC PROPERTIES OF FURTHER STEROIDS<sup>1</sup>

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IT HAS been demonstrated that the polar groups on the steroid molecules influence the renotrophic and androgenic properties of these compounds (1-3). Thus androstenediol  $3\alpha,17\beta$ ,<sup>2</sup> is preferentially renotrophic and  $\Delta^4$ -androstenedione-3,17 is preferentially androgenic. Since the presence of the  $17\beta$  hydroxyl group is essential for maximum renotrophic effect and the  $\alpha,\beta$  unsaturated 3 ketone group for maximum androgenic effect, it seemed worthwhile to determine the activity of steroids containing the various possible polar groups at only one of the two positions, e.g. the 3 and 17. At the same time several interesting new steroids and several relatively inactive steroids at higher dose levels were studied. Finally, a comparison of the effect of these and previously studied steroids on body weight has been made.

## PROCEDURE

Male mice of the Murray-Little dba<sup>3</sup> or the Swiss strain from our Bacteriology Department were castrated under ether anesthesia at 17.0-19.0 grams body weight. One month later pellets of the various steroids<sup>4</sup> were implanted subcutaneously (1).

The food, Rockland checkers, was removed from the mouse cages 18 to 20 hours before autopsy. The mice were killed by decapitation, the organs removed and weighed. The pellets were removed, washed in distilled water, dried and reweighed to determine the amount absorbed.

## RESULTS

*Renotrophic and Androgenic Activities.* The first four steroids in table 1 had been studied previously (1) after implantation of one pellet of the respective compounds. The implantation of four pellets, thus quadrupling the dose level, did not appreciably enhance the potencies of either isoandrosterone or dehydroisoandrosterone; those of androstenedione and androsterone were increased; especially

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<sup>2</sup> The designation of the 17 hydroxyl group has been changed to conform with the recent revision in its steric configuration (4, 5). Thus, the previous  $17\alpha$  hydroxyl group is indicated as  $17\beta$  and cistestosterone as epitestosterone.

<sup>3</sup> These mice were generously supplied by the Biological Station, Springville, New York through the courtesy of Dr. S. G. Warner.

<sup>4</sup> The steroids were generously provided by Ciba Pharmaceutical Products, Inc. through the courtesy of Dr. E. O. Oppenheimer. Many of these compounds were prepared specially for their study by Dr. St. André.

the androgenic activity of androstanedione. It is of interest that although androsterone and androstanedione have similar renotrophic properties, their androgenic potencies are very different.

TABLE 1. RENOTROPHIC AND ANDROGENIC PROPERTIES OF VARIOUS STEROIDS<sup>1</sup>

	MICE No.	STEROID		LIGANDS	REMAINDER + FRACTIONS	TRANS
		Pellet No. <sup>2</sup>	Amount mg/50 day			
Castrated controls	14			(284) <sup>3</sup>	(9) <sup>3</sup>	(35) <sup>3</sup>
17-Methylandrostanol-17 $\beta$ , one-3	3	1:4	0.1	20	170	-51
	4	1:1	0.2	68	1650	-72
	4	1	1.6	93	1450	-83
	4	1	3.3	100	2980	-83
Androstanedione	4	4	32.3	21	1050	-72
Androsterone	4	4	13.0	23	200	-40
Isoandrosterone	4	4	28.2	16	55	-46
Dehydroisoandrosterone	4	4	35.7	10	100	-40
Androstanol-17 $\beta$ , one-3 propionate-17	6	1	0.1	11	78	0
Testosterone-cholesterol carbonate	1	2	0.25	0	-10	
Ditestosterone carbonate	1	2	0.56	0	-10	
17-Ethyl- $\Delta^5$ -androstenediol-3 $\beta$ , 17 $\beta$	1	3	4.4	-6	55	
17-Vinyl- $\Delta^5$ -androstenediol-3 $\beta$ , 17 $\beta$	1	4	1.2	-8	22	
3-Methylandrostanediol-3(?), 17 $\beta$	2	2	8.2	0	-10	-11
3-Methylandrostanol-3(?)one-17	2	1	6.0	6	-10	
$\Delta^5$ -Androstenol-3 $\beta$	2	2	1.5	-9	33	-17
Androstanol-3 $\beta$	2	2	0.3	-1	-22	
Androstanol-3 $\alpha$	2	2	0.4	6	-22	-11
$\Delta^4$ -Androstenone-3	1	2	1.0	-1	-22	8
Androstanone-3	3	2 and 4	pellets sloughed			
Androstanol-17 $\beta$	4	4	0.6	14	46	0
17-Methylandrostanol-17 $\beta$	2	2	1.0	-3	11	-8
Androstanone-17	2	4	0.9	0	11	0

<sup>1</sup> The figures in this column indicate the number of pellets or the steroid: cholesterol composition of the pellet implanted in each mouse.

<sup>2</sup> The values in parentheses are in milligrams, the remainder are percentage difference from these values.

<sup>3</sup> The designation of the 17 hydroxyl group has been changed to conform with the recent revision in its steric configuration (4, 5). Thus, the previous 17 $\alpha$  hydroxyl group is indicated as 17 $\beta$ .

The introduction of the 17-methyl group into androstanol-17 $\beta$ , one-3 did not significantly alter either its renotrophic or androgenic potencies. The propionylation of this steroid, however, rendered it so insoluble (in contrast to testosterone (1-3)) that it was practically inactive. The mono- and ditestosterone

carbonates also were extremely insoluble and inactive. The introduction of either the ethyl or vinyl groups into the 17 position of  $\Delta^5$ -androstenediol-3 $\beta$ ,17 $\beta$  did not make this steroid active. The introduction of the 3-methyl groups into androstenediol-3 $\alpha$ ,17 $\beta$  and androstanol-3 $\alpha$ ,one-17 (androsterone) increased the rate of absorption of these steroids (cf. 1) but obliterated their biological activities.

None of the monosubstituted steroids demonstrated any activity. Furthermore, the rates of absorption from the pellets were extremely low. Androstanol-3 $\alpha$  as well as androstanone-17 also were studied after they were mixed in equal proportions with glucose but the decrease in weight of the pellets at the end of the experiment only indicated an absorption of the glucose (not tabulated). Androstanone-3 was completely insoluble and in three attempts its pellets always sloughed before 7 days of implantation.

Two testosterone derivatives testosterone-n-propyl carbonate and testosterone-17 diethyl amino ethyl carbonate hydrochloride (not tabulated) proved to have approximately the same potency as testosterone. The second compound was not tolerated as a subcutaneous implant presumably because of its acidic nature. It always sloughed out within a few days after implantation.

*Steroids and Body Weight.*<sup>5</sup> Those steroids which demonstrated no renotrophic or androgenic activity (table 1) also did not influence the body weights of the mice. The steroids which were effective are listed in table 2 with steroids previously studied for their renotrophic and androgenic properties.

Androstanol-17 $\beta$ ,one-3; testosterone; androstenediol-3 $\alpha$ ,17 $\beta$  and their derivatives with the exception of 17-ethynyltestosterone, testosterone benzoate and epitestosterone were very effective even at small doses in increasing the body weight of the mice. Several other steroids which are relatively inactive in other effects were able to produce definite increases in body weight when the dose of the steroid was sufficiently great. Notable among these are the androstenediol-3 $\beta$ ,17 $\beta$ ; 17-methyl- $\Delta^5$ androstenediol-3 $\beta$ ,17 $\beta$ ; androsterone, isoandrosterone and dehydroisoandrosterone. Androstenedione and  $\Delta^4$ -androstenedione-3,17 were effective but they also are effective in other biological activities (1-3). None of the pregnene and pregnane compounds affected body weight. It should be noted also that these last mentioned steroids are not very soluble in tissue fluids.

#### DISCUSSION

It is obvious now that the androgenic steroids have several physiological properties which do not quantitatively parallel each other (2, 3). Thus, the presence of the 3 $\alpha$  hydroxyl group reduces the androgenic but not the renotrophic properties and the 3 keto group enhances the androgenic without increasing the renotrophic properties of these steroids. This effect of chemical structure is noted even in steroids of very low activity, e.g. androsterone versus androstenedione. In spite of this divergence in properties as yet none of the steroids has shown complete dichotomy in these two physiological activities. An attempt to produce this by the production of steroids with only one polar group has been unsuccessful.

<sup>5</sup> Parts of these data have been previously reported in the Josiah Macy Jr. Foundation Reports on Metabolic Aspects of Convalescence, 6th meeting, New York 1944 and in a review (2).

TABLE 2. EFFECT OF STEROIDS<sup>1</sup> ON BODY WEIGHT OF CASTRATED MOUSE

	MICE NO.	STEROID		CHANGE IN BODY WEIGHT	
		Tablet No.	Absorbed mg./100 days	gm./100 days	mg.
Castrated controls	43			1.7	
Androstanol-17 $\beta$ , one-3	5	111	0.65	5.6	112
	6	1	2.6	4.8	181
	3	3	7.2	3.4	100
Androstanol-17 $\beta$ , one-3, prop.-17	6	1	0.1	1.6	-6
17-Methylandrostanol-17 $\beta$ , one-3	3	114	0.1	2.9	70
	4	111	0.7	3.7	118
	4	1	1.6	3.6	111
	4	2	3.3	3.5	106
Testosterone	4	114	0.14	3.4	100
	4	111	1.15	3.4	100
	6	211	2.1	3.9	120
	9	1	8.5	4.2	159
	4	2	16.6	4.8	182
Testosterone propionate	4	112	0.2	4.3	153
	4	111	0.88	4.1	141
	4	211	2.33	3.5	106
	11	1	4.4	4.3	153
	5	2	9.4	3.4	100
Testosterone-acetate-3, propionate-17	2	1	1.3	4.8	182
17-Methylandrostanediol-3 $\beta$ , 17 $\beta$	5	1	0.4	3.0	76
	6	3	1.8	3.1	82
17-Ethynylandrostanediol-3 $\beta$ , 17 $\beta$	5	1	0.5	2.9	70
$\Delta^5$ -Androstenediol-3 $\beta$ , 17 $\beta$	3	1	0.7	2.3	35
17-Methyl- $\Delta^5$ -Androstenediol-3 $\beta$ , 17 $\beta$	6	1	0.9	2.4	41
	4	4	2.4	4.5	164
17-Ethynyl- $\Delta^5$ -androstenediol-3 $\beta$ , 17 $\beta$	5	1	1.0	2.5	47
3-Methyl- $\Delta^3, 5$ -androstadienol-17 $\beta$	6	1	5.7	2.0	18
3, 17-Dimethyl- $\Delta^3, 5$ -androstadienol-17 $\beta$	6	1	2.6	2.4	41
Androsterone	6	1	3.7	2.3	35
	4	4	13.0	4.6	171
Isoandrosterone	8	1	9.3	2.8	65
	4	4	28.2	3.3	94
Etiocholanol-3 $\alpha$ , one-17	1	1	7.9	1.8	6
Dehydroisoandrosterone	5	1	10.9	4.4	159
	4	4	35.7	3.6	112

TABLE 2 (Continued)

	MICE NO.	STEROID		CHANGE IN BODY WEIGHT	
		Pellet No. <sup>1</sup>	Absorbed mg/30 days	gm/30 days	% <sup>2</sup>
Androstanedione	7	1	9.2	3.9	129
	4	4	32.3	4.3	153
$\Delta^1$ -Androstenedione-3, 17	6	1	10.6	3.9	129
	2	2	19.9	3.3	94
17-Methyltestosterone	4	1:4	0.14	5.1	200
	4	1:2	0.57	3.1	82
	5	1:1	1.30	3.3	94
	6	1	8.5	4.8	182
	4	2	14.4	4.1	141
17-Vinyltestosterone	8	1	8.1	4.4	159
17-Ethyltestosterone	9	1	5.1	3.4	100
17-Ethynyltestosterone	6	1	0.5	2.0	18
Testosterone benzoate	6	1	0.2	2.2	29
Epitestosterone	4	1	3.6	1.9	12
Androstenediol-3 $\alpha$ , 17 $\beta$	2	1:1	0.25	4.8	182
	11	1	1.7	4.5	165
	3	4	5.9	3.9	129
Androstenediol-3 $\alpha$ , 17 $\beta$ , acetate-3	3	1	0.7	3.5	106
	6	3	2.3	3.5	106
	5	4	3.0	4.6	171
17-Methylandrostanediol-3 $\alpha$ , 17 $\beta$	5	1:1	0.6	4.0	135
	7	1	2.6	4.1	141
	4	2	3.2	4.2	147
	5	4	6.9	4.3	153
Androstenediol-3 $\beta$ , 17 $\beta$	6	1	0.2	3.4	100
	4	4	2.0	3.9	129
Progesterone	5	1	5.6	2.1	24
$\Delta^5$ -Pregnenol-3 $\beta$ , one-20	5	1	0.8	1.2	-29
Allopregnanol-3 $\alpha$ , one-20	2	1	0.1	1.9	12
Pregnanol-3 $\alpha$ , one-20	1	1	2.9	1.2	-29

<sup>1</sup> The figures in this column indicate the number of pellets or the steroid: cholesterol composition of the pellet implanted in each mouse.

<sup>2</sup> Percentage difference from the castrated controls.

<sup>3</sup> The designation of the 17 hydroxyl group has been changed to conform with the recent revision in its steric configuration (4, 5). Thus, the previous 17 $\alpha$  hydroxyl group is indicated as 17 $\beta$ .

These compounds did not have any physiological activity due at least in part, to their very low solubility in the tissue fluids. Thus, the presence of a second polar group is essential for solubility and very likely the physiological activity of the steroids.

It has been previously (1) suggested that the physiological and biochemical properties of the androgens may depend not only upon the steroid administered but also the nature and number of metabolites formed by the various tissues of the body (6, 7).

The increase in body weight produced by the various steroids is in agreement with the protein anabolic properties of these androgens (3) except that several of the steroids (e.g. androsterone, dehydroisoandrosterone) which are relatively inactive in all physiological properties produced significant increases in body weight. This may be a matter of dosage; only trace amounts of the most active compounds were needed to produce similar effects. On the other hand, it is probable that these compounds may influence phases of growth other than protein anabolism, e.g. fat, water. Preliminary nitrogen balance studies in rats indicate that androsterone stimulates greater increases in body weight than can be accounted for by changes in nitrogen excretion.

#### SUMMARY

Since previous studies had indicated that the maximum renotropic and androgenic properties of steroids implanted as pellets in castrated mice were dependent upon the nature of the chemical groups of the molecule, a series of C-19 steroids with polar groups only in the 3 or 17 position were studied. All of these monosubstituted steroids demonstrated no solubility or only a trace in the tissue fluids and also no biological activity.

A comparison of the effect of these and previously studied steroids on the body weight indicated that many steroids which had only trace or small renotropic and androgenic effects were able to appreciably increase the body weight of the mice.

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# INFLUENCE OF PREVIOUS DIET ON METABOLISM DURING FASTING<sup>1</sup>

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THE metabolism of the rat both during feeding and subsequent fasting has been demonstrated to be dependent upon the fat, carbohydrate, and protein content of the preceding diet (cf., reviews by Roberts and Samuels (1) and Samuels (2)). In general, it appears that the foodstuff mainly burned under these conditions corresponds to the predominant source of energy in the diet; this phenomenon has been termed 'preferential utilization' (1). The adaptation to diet which occurred was shown to develop in the peripheral tissues as well as in the liver and kidney (3, 4), and to be relatively independent of the secretion of the adeno-hypophysis (5).

In the present investigation, it was observed that rats previously maintained on a diet high in fat survived longer during subsequent fasting than similar animals previously fed a high carbohydrate diet (cf., also, 6). This difference was not associated with any significant difference in voluntary activity in the 2 groups during the early fasting period. The results presented below suggest that the difference in survival time was due primarily to a more rapid rate of protein breakdown in the high carbohydrate group.

## METHODS

Adult, male rats, obtained from Sprague-Dawley, Inc., and previously maintained on Purina Fox Chow, were force-fed the special diets by stomach tube for a period of 6 weeks prior to fasting. Equicaloric amounts of the high fat and high carbohydrate diets were fed to groups of animals of similar age and weight. These diets contained approximately 85 per cent of their calories in the form of the major foodstuff and 15 per cent as lactalbumin, supplemented with all known dietary essentials. The procedures used, as well as the constitution of the diets, have been described in detail elsewhere (7, 8). During the feeding and fasting periods, drinking water was allowed *ad libitum*.

The animals were kept in individual metabolism cages (*Group I*) or in individual activity cages (*Group II*) during the entire experimental period. In the former instance, urine collections were made usually at weekly intervals. These samples were acidified and preserved under toluene during collection and, at a later time, were analyzed for total nitrogen by a modification of the macro

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Kjeldahl method (9), and for acetone bodies by the Van Slyke procedure (10). During the fasting period, occasional determinations were made of the number of cage rotations, and the blood sugar level for a number of the animals kept in the activity cages. Only complete rotations were registered on the comptometer in the apparatus used, so that an indication of the energy required to move the activity cage less than a full rotation was not obtained. Tail blood samples were analyzed for total sugar by the Reinecke method (11). Body weights were followed at frequent intervals for several animals in both *Groups I* and *II*.

At the time of death, a small portion of the liver was removed (about 500 mg.) and saved in 50 per cent  $H_2SO_4$  for later analysis of total nitrogen by the Kjeldahl procedure (9). The remainder of the liver was dropped into 95 per cent ethanol and later analyzed for fat by a method previously described (5). Also at autopsy, the left kidney of the animals in *Group I* was secured for nitrogen determination, and the right kidney for fat analysis by the methods described.

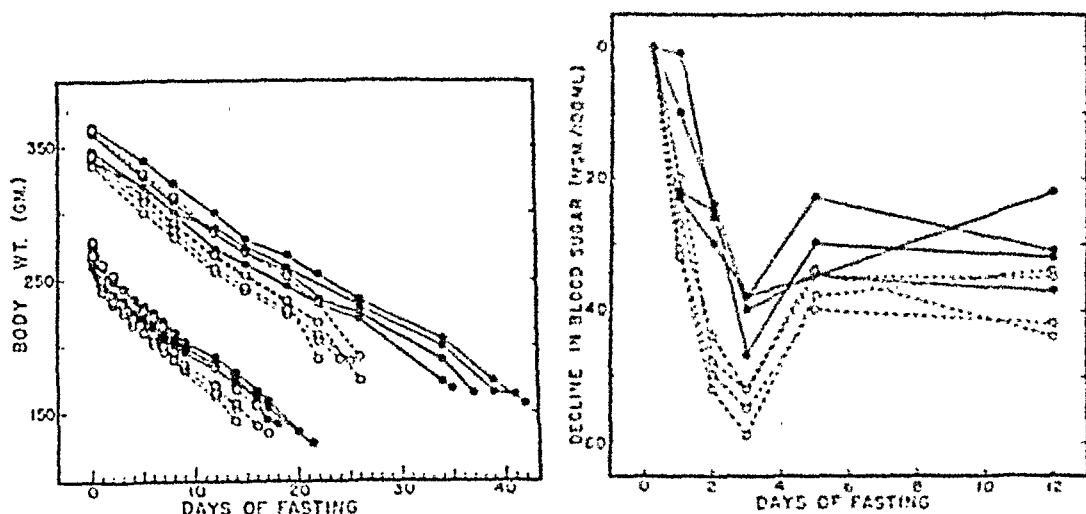


Fig. 1 (left). INFLUENCE OF PREVIOUS DIET ON body weight loss and survival time during fasting. All animals had previously been force-fed for 6 weeks either the high carbohydrate diet (open circles) or the high fat diet (solid circles).

Fig. 2 (right). INFLUENCE OF PREVIOUS DIET ON the decline in blood sugar during the early fasting period. All animals had previously been force-fed for 6 weeks either the high carbohydrate diet (open circles) or the high fat diet (solid circles).

## RESULTS AND DISCUSSION

The influence of previous diet on the loss in body weight and on the survival time during fasting is shown in figure 1. In two series of rats of different age and weight ranges, those animals previously fed a high fat diet (solid circles) lost weight more slowly and survived longer than the group maintained on the high carbohydrate diet prior to fasting (open circles). As might be expected, the higher the initial body weight at the time of food deprivation, the longer the period of survival. Thus, in *Group I*, consisting of animals whose body weight was approximately 350 gm. at the time the fast was instituted, the mean survival time was 24.6 days in the case of those rats previously fed the high carbohydrate (CHO) diet, and 38.8 days after the high fat diet (cf. table 1). The mean survival time for animals in *Group II*, comprised of rats weighing approximately 270 gm. at the beginning of fasting, was 15.3 days for the high carbohydrate group, and 19.7 days for the high fat group. It may be noted that the average percentage of body weight lost before death supervened was similar

for both series of animals maintained on similar diets: 45.5 per cent and 45.8 per cent after a high carbohydrate regime in *Groups I* and *II*, respectively; and 54.3 and 51.7 per cent after the high fat diet.

The voluntary activity of the animals in *Group II* was quite variable throughout the fasting period and exhibited a pre-mortal rise in all animals (cf. also 6). Although those animals previously on the high carbohydrate diet tended to be more active than the fat-fed rats, the differences observed were not significant during the early fasting period. Thus, in the first week of fasting, the number of cage revolutions per day recorded for 7 rats previously on the high fat diet was  $106 \pm 32$  (mean  $\pm$  standard error). The corresponding figure for 5 animals in the high carbohydrate group was  $194 \pm 76$ . After the first week, the animals in the latter group were definitely more active; in several cases, this increase in voluntary activity appeared to be associated with approaching death. The premortal rise in activity was also shown by the rats previously fed fat, but at a later time. A similar difference, in part due to the earlier

TABLE 1. INFLUENCE OF PREVIOUS DIET ON THE COMPOSITION OF LIVER AND KIDNEY IN RATS FASTED TO DEATH<sup>1</sup>

PREVIOUS DIET	NO. OF RATS	SURVIVAL TIME	INITIAL FASTING BODY WT.	TOTAL FASTING WT. LOSS	LIVER WT.	LIVER NITRO-GEN	LIVER LIPID	KIDNEY WT.	KIDNEY NITRO-GEN	KIDNEY LIPID
		days	gm.	%	gm/100 gm.			gm/100 gm.		
<i>Group I</i>										
High CHO.....	6	$24.6 \pm 0.6$	$343 \pm 5$	$45.5 \pm 1.1$	$1.17 \pm 0.04$	$39.1 \pm 1.2$	$30.5 \pm 3.4$	$0.79 \pm 0.02$	$24.4 \pm 0.8$	$34.1 \pm 2.8$
High fat.....	5	$38.8 \pm 2.7$	$352 \pm 6$	$54.3 \pm 1.0$	$1.23 \pm 0.08$	$39.2 \pm 2.1$	$35.7 \pm 4.3$	$0.85 \pm 0.03$	$25.4 \pm 0.8$	$33.6 \pm 6.9$
<i>Group II</i>										
High CHO.....	8	$15.3 \pm 0.4$	$272 \pm 3$	$45.8 \pm 0.8$	$1.44 \pm 0.06$	$47.5 \pm 2.0$	$21.7 \pm 1.1$	$0.89 \pm 0.02$		
High fat.....	9	$19.7 \pm 0.8$	$265 \pm 3$	$51.7 \pm 0.9$	$1.36 \pm 0.09$	$46.0 \pm 2.4$	$22.0 \pm 4.5$	$0.80 \pm 0.07$		

<sup>1</sup> The values shown represent the mean  $\pm$  standard error, and are expressed as mg/100-gm. body weight, except where noted otherwise.

premortal rise in the carbohydrate-fed rats, was observed in the exercise and fasting experiments of Samuels *et al.* (6). The differences in survival time and metabolic behavior, however, were greater than can be accounted for by the differences in activity. It may also be noted, in passing, that no significant difference was detected in the basal metabolic rates of similar rats kept on these diets (3).

The sparing of the carbohydrate stores of the body in fasted animals previously fed a high fat diet has already been reported; this was manifested in the earlier studies by a slower disappearance of liver glycogen (12) and a slower fall of blood sugar under a wide variety of conditions (3, 4, 6). In the present investigation this sparing action was again seen in the slower decline in blood sugar in the fat-fed animals of *Group II*, as compared to that observed in animals fasted after maintenance on a high carbohydrate regime (fig. 2). Higher levels of blood sugar were present in the fat-fed group throughout the early fasting period. After the first week of fasting the blood sugar levels tended to be similar in both groups. The level of the blood sugar toward the end of the second week averaged approximately 60 mg/100 ml. in the high carbohydrate group and about 65 mg/100 ml. in the high fat group. Although the blood sugar levels were not followed beyond the end of the second week, it has been found

in similar studies that the differences tend to disappear at this time, and that, terminally, the levels increase sharply in both groups of animals (6).

The shape of the blood sugar curves during fasting indicates that soon after initiation of the fast, the metabolic requirements of all animals were probably being supplied largely from non-carbohydrate sources. The carbohydrate stores have been shown to be depleted within 48 hours of fasting in a similar series of animals previously fed high fat and high carbohydrate diets (12). The upswing in the blood

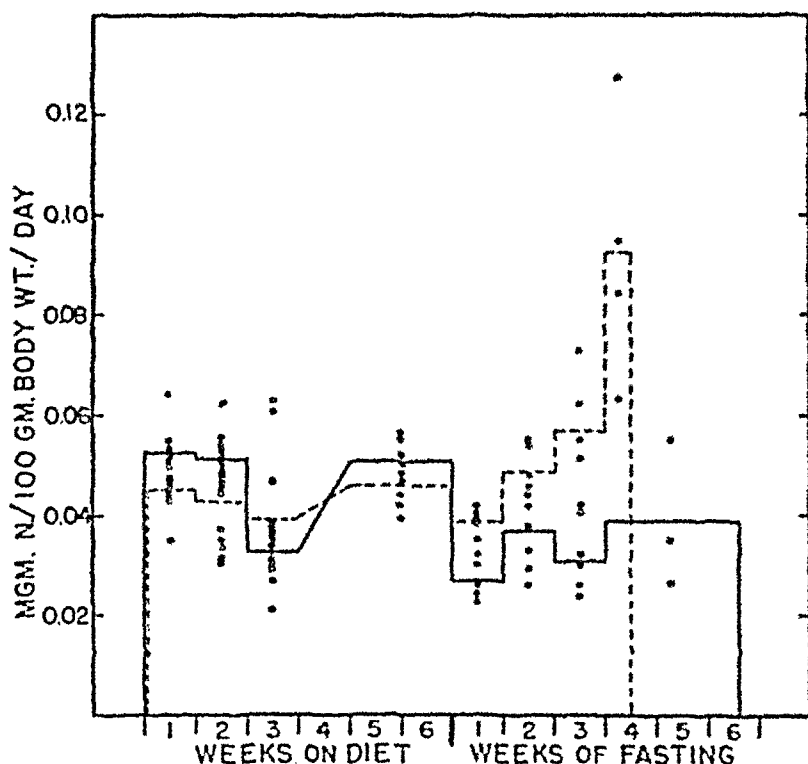


Fig. 3. INFLUENCE OF DIET ON the urinary excretion of nitrogen during feeding and subsequent fasting. The dotted line represents the average excretion for animals fed the high carbohydrate diet; individual values are indicated by open circles at the middle of each collection period. Similarly, the solid line and solid circles represent the averages and individual excretion values for animals maintained on the high fat diet.

sugar curve in both types of animal 3 days after removal of food suggests a sparing of carbohydrate by the accelerated utilization of other energy sources. As will be demonstrated below, fasting apparently resulted in an increased catabolism of protein in the carbohydrate group and an augmented metabolism of fat in the fat-fed animals.

During the feeding period, both dietary groups of animals excreted approximately the same amount of nitrogen in the urine (fig. 3). This indicates that the level of protein catabolism (and anabolism) was similar in both groups during feeding. Soon after initiation of the fast, however, the carbohydrate-fed animals began to excrete significantly more nitrogen than the fat-fed group. Premortally, very large amounts of nitrogen, larger than excreted during the feeding period, were found in the urine of the former group. Since the blood sugar level of a fasting animal would

presumably be rapidly reduced unless replenished from some other source or spared by the utilization of some other foodstuff, it appears likely that the carbohydrate-fed animal was not only obtaining its energy from protein sources, but that gluconeogenesis was also proceeding at a substantial rate even during the later fasting period.

The relative sparing of carbohydrate exhibited by the fat-fed rats was apparently due to a higher rate of fat utilization. Figure 4 reveals that the urinary excretion of acetone bodies was higher in this group than in the carbohydrate-fed group throughout the entire feeding and fasting periods. At the institution of the fast, a

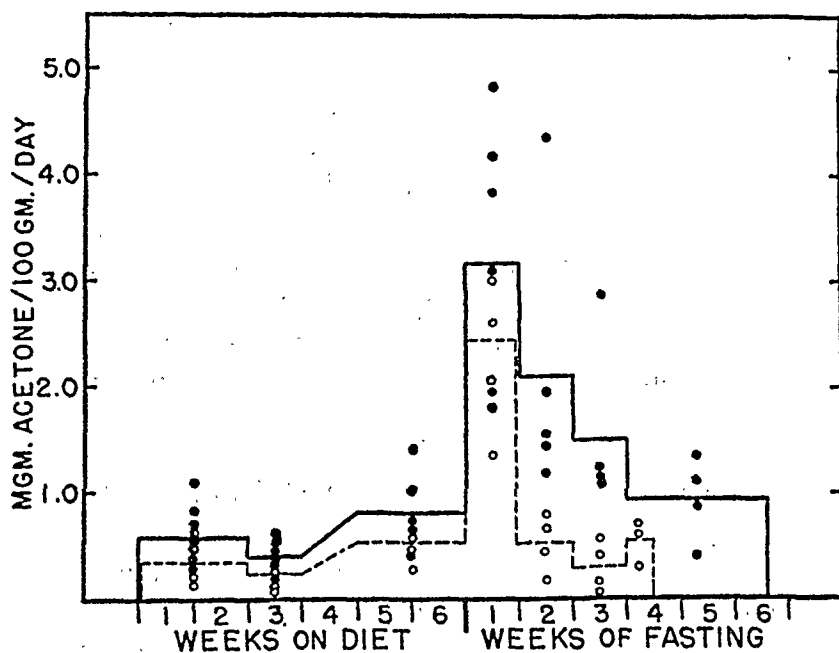


Fig. 4. INFLUENCE OF DIET on the urinary excretion of acetone bodies during feeding and fasting. The dotted line represents the average excretion for animals fed the high carbohydrate diet; individual values are indicated by open circles at the middle of each collection period. Similarly, the solid line and solid circles represent the averages and individual excretion values for animals maintained on the high fat diet.

very high rate of acetone body excretion was seen in both groups, but this was maintained beyond the first week only in the high fat group. In the latter group, moreover, the fasting increase in acetone body excretion was associated with a decrease in the excretion of nitrogen.

The influence of previous diet on the nature of the metabolic mixture used during fasting is also delineated by the results in table 1. It has already been shown that previous fat feeding increases the storage of fat in the liver and carcass of the rat (12), but has no effect on kidney lipid (4), or on liver, kidney, or carcass nitrogen (4, 5). In the present investigation, previous diet resulted in no significant differences in any of these values which were measured at the time of death. Since the high fat animals survived longer, organ nitrogen was apparently spared at the expense of organ fat. The relative levels of nitrogen and acetone body excretion in the two dietary groups during the fasting period permit a similar conclusion for the over-all metabolism of the animals.

The results indicate that those animals previously fed the diet high in fat became adapted to the metabolism of fat during the feeding period. This was evidenced by the higher level of acetone body excretion during this time. This preferential utilization of fat continued in this group throughout the subsequent fasting period, as shown by the maintenance of a high level of acetone body excretion and a sparing of carbohydrate and protein. In the carbohydrate-fed group, however, the utilization of carbohydrate for energy was apparently accelerated during feeding, and was kept at a high level after initiation of the fast. The carbohydrate stores in these animals would presumably be depleted rapidly during fasting, were not energy forthcoming from some other endogenous source. The results demonstrate that protein catabolism supplied this requirement. It cannot be determined from the present data what portion of this protein was converted to glucose before being burned. The shape of the blood sugar curves suggests, however, that gluconeogenesis occurred to some extent throughout the fasting period in both groups of animals.

The longer survival time of the high fat animals during fasting was, in all likelihood, related to the sparing of tissue protein by the continued utilization of fat and a belated reduction in spontaneous activity. As a result, the protein requirements of the organism for the production and maintenance of essential cellular constituents were not as rapidly depleted in the high fat group as in the high carbohydrate group.

#### SUMMARY

The ability of the rat, previously force-fed a high fat diet for 6 weeks, to survive longer during fasting than similarly treated animals previously maintained on a high carbohydrate regime, appears to be related to the capacity of the former preparation to continue burning mainly fat for energy during the fasting period, thus sparing carbohydrate and protein stores. This was evidenced by an enhanced excretion of acetone bodies in the fat-fed group, a slower disappearance of blood sugar, and a lowered rate of nitrogen excretion after initiation of the fast. On the other hand, the apparent rapid depletion of carbohydrate stores in the carbohydrate-fed animal after the deprivation of food, as indicated by the rapid early decline in blood sugar, was followed by increased utilization of protein for energy. It may be deduced that the earlier death of the animals in the latter group was associated with a more rapid depletion of body protein to the point where there were no longer sufficient quantities of this substance available to satisfy the minimal requirements of the organism.

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# MECHANISMS OF METABOLIC INHIBITION BY HEAVY METALS<sup>1</sup>

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PREVIOUS reports on the electrochemical properties of the synovialis have distinguished at least two types of membrane or boundary potentials. With many ions the potentials are of the magnitude predicted by the theory of diffusion potentials, and in a considerable number of cases are highly correlated with ionic mobilities in aqueous solution (1). Potentials of this type may be conveniently termed Planck potentials. In distinct contrast with these are the potentials produced at the internal surface of the synovialis by a large number of metabolically active substances, including cytochrome inhibitors, heavy metals and thiols. The high potentials occurring in the presence of low concentrations of metabolic inhibitors are not explained by the Planck or Henderson theories of diffusion potentials. Evidence has been presented that the effects are related to changes in the source or terminal potentials of the cytochrome system. Specifically, the hypothesis was made that the change of potential measured was directly related to the average difference of potential between the electron acceptor components (cytochromes *b* and *c*) and the electron discharging, or terminal component, normally cytochrome oxidase. Under the experimental conditions, many of the potentials were reversibly reproduced by aspirating and replacing the solutions in the joint cavity. The potentials thus evidently are produced at the inner bounding surface of the cavity. That the difference of potential in the cytochromes of the surface structures is the determining factor was evident from the relation of the potentials to known effects of the agents on enzymes (2).

As a continuation of the preceding studies, observations have been made on the effects produced by certain heavy metals introduced into the knee joint cavity as chlorides in isotonic (0.15 M) NaCl solutions. The original observations, which were restricted to concentrations giving maximal potentials, have been extended to include a wide range of concentrations. The object of the experiments is to obtain a detailed comparison of the effects of five heavy metals; copper, iron, mercury, gold, and cobalt, over a wide range of concentrations. This can be regarded as a necessary first step in working out the mechanisms of heavy metal activation and inhibition.

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<sup>2</sup> The data in this paper are included in a thesis in partial fulfillment of the requirements for the degree of Master of Science, 1949.

## EXPERIMENTAL RESULTS

All experiments were performed on the knee joints of dogs anesthetized by intra-peritoneal injection of 3 per cent sodium pentobarbital, a dosage of 0.5 cc. per pound body weight being given. Approximately equal numbers of normal male and female animals were studied. Synovial membrane potentials were measured by the modified method described in the preceding paper (2). In all instances the liquid junction was made with 0.15 M NaCl in the subcutaneous tissue connected to the reference calomel electrode by means of a saturated KCl bridge. By means of a saturated KCl agar bridge, junction was made at the indicator intra-articular electrode with dilute neutral

TABLE 1. EFFECTS OF HEAVY METAL IONS ON SYNOVIAL POTENTIALS

CONCENTRATION OF METAL ION, MOL./L.	AVERAGE POTENTIALS IN MILLIVOLTS <sup>1</sup>				
	Cu <sup>++</sup>	Fe <sup>+++</sup>	Hg <sup>+++</sup>	Au <sup>+++</sup>	Co <sup>++</sup>
$7.5 \times 10^{-10}$	7	-12	2	17	—
$7.5 \times 10^{-9}$	5	-3	4	15	—
$7.5 \times 10^{-8}$	0	-0	13	25	—
$7.5 \times 10^{-7}$	4	-8	1	18	2
$7.5 \times 10^{-6}$	13	-6	11	24	2
$7.5 \times 10^{-5}$	55	-1	30	56	3
$7.5 \times 10^{-4}$	146	67	180	231	74
$7.5 \times 10^{-3}$	139	149	102	87	14
No. experiments. ....	8	5	4	4	5

<sup>1</sup> Potentials produced at articular surface of the synovial membrane by metallic chloride in isotonic NaCl solution. The initial stable NaCl potential is taken as zero reference point.

solutions of heavy metal chloride in isotonic NaCl. The sign of the potential is taken as that of the indicator electrode connected to the internal boundary of the joint cavity.

The effects of Cu<sup>++</sup>, Fe<sup>+++</sup>, Hg<sup>+++</sup>, Au<sup>+++</sup>, and Co<sup>++</sup> ions in various concentrations were determined. The value of the resting potential difference across the synovialis was determined with 0.15 M NaCl in the joint cavity. The synovial cavity was aspirated and washed with this solution until a sufficiently constant reading was obtained. The cations were employed in concentrations increasing exponentially by a factor of 10 from  $7.5 \times 10^{-10}$  M to  $7.5 \times 10^{-3}$  M. The lowest concentration of each salt was first studied by application to the intra-articular surface, readings being taken for one minute of exposure. At the end of one minute the solution was replaced and two additional readings were taken at half minute intervals. Average values for the four readings were computed. Before application of the heavy metal solution of next highest concentration, the joint cavity was flushed three times with 0.15 M NaCl, potential readings being taken with each replacement. The average values of these three readings afford criteria as to the stability of the heavy metal potentials.

With the original stable NaCl level as a base of reference, the average values obtained for the 5 heavy metal chlorides are presented in table 1. Four representative

experiments are shown in figures 1 to 4, in which the stabilities of the potentials are indicated by comparison with curves representing the average potential of 3 successive replacements with pure isotonic NaCl. It is clear from the figures that the NaCl values are roughly parallel to those obtained with the test solution. The high positive readings were brought down to the original NaCl level only after repeated rinsings with isotonic NaCl. In many instances, the original level could not be restored with NaCl unless an effective thiol reducing agent such as glutathione is added in about 0.01 M concentration. The heavy metal effects differ in this respect from those of many other inhibitors such as cyanide, iodide, sulphide, etc., which are easily reversible with isotonic NaCl (2).

Inspection of the curves obtained by plotting logarithms of metal concentration against the observed potentials reveals certain similarities with respect to all the

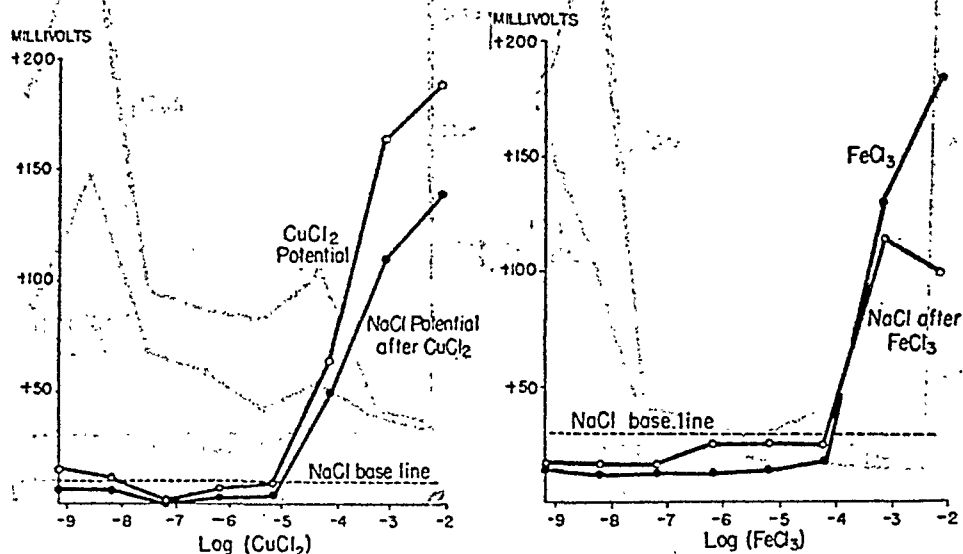


Fig. 1 (left) EFFECT OF CUPRIC IONS ON potential

Fig. 2 (right). EFFECT OF FERRIC IONS ON potential

metal ions. The curves for all but gold are relatively flat at concentrations up to about  $10^{-5}$  or higher. With mercuric and auric ions, a maximal positivity was attained in the neighborhood of  $10^{-3}$  M. Copper also showed maximal positivity at about that concentration, but the maximum was far less sharp, and did not always occur. Cobaltous ion showed a definite maximum in the same region but the effect was much smaller than that obtained with ions in the higher state of oxidation. Because of its great instability, cobaltic ion has not been studied. At concentrations lower than  $7.5 \times 10^{-5}$ , ferric ions consistently yielded potentials more negative than the NaCl base line. Copper and mercury occasionally showed this effect at very low concentrations, but on the average produced slightly positive potentials.

While the experimental results showed consistent similarities in the potential-concentration curves of all the heavy metals except cobalt, they also revealed idiosyncracies characteristic of each metal. For example, at all concentrations up to the highest, gold yielded the most positive potential, but at the highest concentration,  $7.5 \times 10^{-3}$  M it was the lowest in the series. In contrast with all the others, gold exerted high positivity at very low concentrations. The behavior of iron was in decided

contrast, for it was highest in the series at the highest concentration,  $7.5 \times 10^{-3}$  M, but lowest at concentrations of  $7.5 \times 10^{-4}$  and less. Cobalt was not comparable, for it had been studied only in its state of lower valence. Mercury and copper fell between iron and gold at all concentrations, with mercury showing a sharp maximum at  $7.5 \times 10^{-4}$  M, at which concentration copper reached a relatively flat maximum. At the lower concentrations, iron was consistently negative, gold consistently positive, while copper and mercury produced either low positive or negative potentials, the average in both cases being slightly positive.

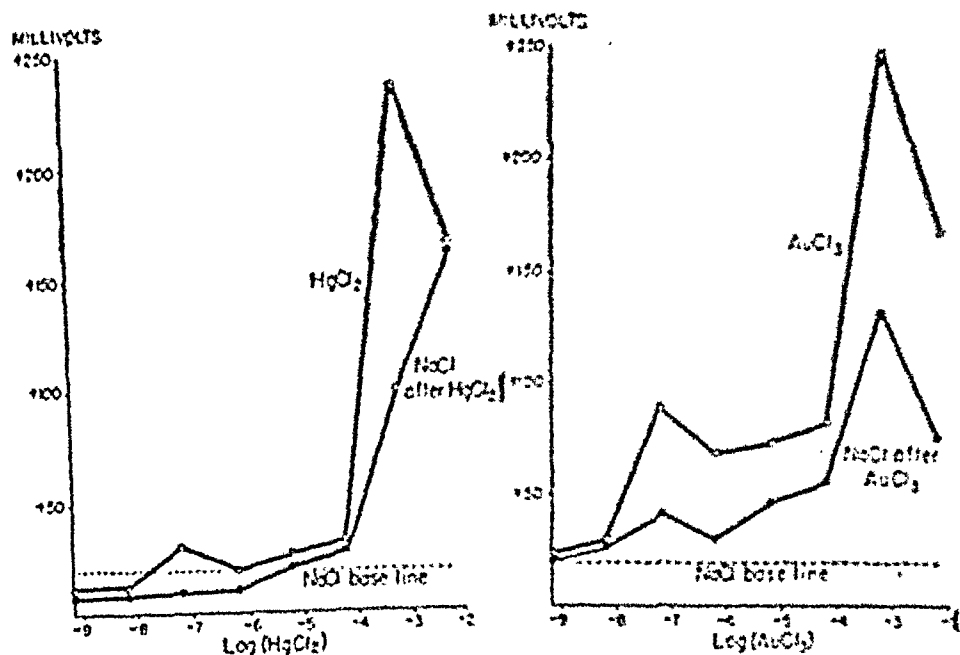


Fig. 3 (left). EFFECT OF MERCURIC IONS ON POTENTIAL

Fig. 4 (right). EFFECT OF AURIC IONS ON POTENTIAL

With respect to stability of the potentials there were also consistent and characteristic differences among the metals. The least stable potentials were those produced by gold, especially at the highest concentration,  $7.5 \times 10^{-3}$  M. At this concentration, it typically showed a very high initial potential of 400 to 500 mv., but this fell very rapidly, within 5 or 10 seconds, to a comparatively stable level. At this level readings taken over a period of one minute agreed consistently with those taken under similar conditions during a second washing with the solution containing gold. Iron is the only other metal which showed a tendency for the potential to decrease from its initial value. However, the initial potential, unlike that of gold, was found to be of approximately the same magnitude as the more stable potentials obtained by longer exposure. Copper and mercury showed less tendency to give unstable potentials than either of the others. Cobaltous ions gave appreciable potentials only at the higher concentrations,  $7.5 \times 10^{-4}$  M and above, and these potentials tended to drift upward.

With respect to stability of the potentials after rinsing with isotonic NaCl, there were equally characteristic differences. With this as a criterion, mercury, copper

and iron appeared to be most firmly fixed in the tissues, while gold was more easily removed by rinsing with saline solution. An apparent anomaly sometimes occurred at concentrations higher than that which produced the maximal potential. This is illustrated for mercury in figure 3. When the solution containing  $\text{HgCl}_2$  at  $7.5 \times 10^{-3} \text{ M}$  was replaced with  $\text{NaCl}$ , the potential fell only slightly. When, however, the  $7.5 \times 10^{-4} \text{ M}$  solution was replaced, the considerably higher potential fell to a low level. It seems evident from the nature of the potential-concentration curve in figure 3 that removal of approximately the same fraction of inhibitor in each case could lead either to a sharp drop in the potential or to only a slight change. The stability of the potential with washing depends, therefore on at least 2 factors: the stability of the complexes formed by the metal in the tissues, and the slope of the potential-concentration curve in the region in question. If the slope in this region is negative, i.e., if the potential falls as the concentration is increased, then removal of a fraction of the inhibitor may actually result in an increased potential, which may approach the maximal potential.

It is clear that for any of the heavy metals the ratio of free to fixed metal depends on the total concentration. For example, it would appear from the shape of the curves for iron (fig. 2) that this ratio is high at the highest concentration, for the potential at this point falls by almost 50 per cent after the rinsing with  $\text{NaCl}$ , as compared with about a 10 per cent change at the next lower concentration. An effect of this kind would be predicted as a consequence of saturation of chemical groups available for complex formation.

#### DISCUSSION

Attempts to explain the origin of the positive or negative potentials produced by contact of heavy metals with the synovial tissues depend on a general theory of tissue potentials. As a working hypothesis, it has previously been proposed that the effects are brought about by changes of potential in the cytochrome system at which electrons are transferred to the cytochrome acceptor component (source potential) or discharged to oxygen or another terminal electron acceptor (terminal potential) (2). The effects have been referred to the cytochromes for the following reasons: They are distinguished from other enzymes by the fact that they are electron acceptors and donors. Electrons combine with the ferric forms of the enzymes, forming hydrogen ions (protons) from hydrogen donors. The electrons are transferred to oxygen, the terminal electron acceptor, via cytochrome oxidase, the component of the system which has a redox potential corresponding to that of a rather strong oxidant (3). Cytochrome *b* and cytochrome oxidase, unlike most of the components of hydrogen transport mechanisms, occur predominantly in the solid structures of cells. Accordingly electrons are conducted along a potential gradient in the solid phase. Electrochemically the system may be supposed to function in some ways analogously to a platinum electrode connected to 2 solutions of different redox potentials. In reversible electrode reactions hydrogen may be split into protons and electrons. By conduction to the other solution the electrons combine with oxidants such as protons, the higher valence states of metallic ions, or oxygen. In the case of the cytochrome system the terminal electron acceptor is normally oxygen.

On the basis of this hypothetical mechanism, electrochemical considerations have led to an approximate formulation for observed potential differences in the presence of inhibitors or activators (1)

$$E_{\text{obs}} = \bar{E}_{s,1}^0 - \bar{E}_{t,1}' \quad (1)$$

where  $\bar{E}_{s,1}$  is the mean difference of potential between source and terminal in the cytochrome system.  $\bar{E}_{s,1}^0$  refers to the unaffected, normal side of the biological membrane, while  $\bar{E}_{t,1}'$  refers to the side on which the inhibitor or activator is applied. In applying this formula, it is convenient to assume that  $\bar{E}_{s,1}^0$  is constant. Observed potentials are thus assumed to depend essentially on mean differences of potential between the source and terminal cytochrome components, the potentials of which vary in the presence of activators or inhibitors.

On the basis of this hypothesis, the effects of a large number of active substances have been explained either as known direct effects on the cytochrome system, or as effects on hydrogen transport systems or enzymes coupled with the cytochromes. Accordingly inhibitors or activators are to be classified as to their action on source or terminal potentials. They may act directly on one of the cytochromes, as for example cyanide or sulphide, or they may act primarily on a hydrogen transport system. By blocking or facilitating metabolic pathways, reagents may change the source potential at which dehydrogenation is coupled with the splitting of electrons from protons at the cytochrome level. Equation 1 becomes inaccurate when there is appreciable ionic conductance as well as electron conduction across the membrane. Theoretically it is accurate only for boundaries without ionic transference. The effect of increasing ionic conductance is to lower the observed potential, the source and terminal potential being constant. Consequently, potentials calculated theoretically from known levels in the cytochrome system are upper limits which observed potentials can approach when ionic transference is negligible.

In applying equation 1 to the experimental results, it is convenient to break  $\bar{E}_{s,1}$ , the variable term, into 2 terms, representing the mean source potential and the mean terminal potential.

$$\bar{E}_{s,1} = \bar{E}_s' - \bar{E}_t' \quad (2)$$

Thus, according to equations 1 and 2, an increased source potential,  $\bar{E}_s'$ , results in a positive observed potential, while an increased terminal potential,  $\bar{E}_t'$ , has the opposite effect.

On the basis of these considerations, the characteristics of the heavy metals may be considered. Gold differs from the others by yielding a relatively high potential at concentrations as low as  $10^{-7}$  M. As with ferric and mercuric ions there is a sharp increase in positivity in the region of  $10^{-4}$  M. Further, as with mercury, there is a sharp maximum in the region of  $10^{-3}$  M. Gold may be distinguished from the others by the fact that the trivalent auric ion is reduced by accepting 2 electrons. This would require trimolecular collisions with reducing agents that yield one electron. Considered as an oxidation-reduction system, auric-aurous ions are at the oxidative extreme of the systems studied; the auric ion is capable of oxidizing ferrous, cuprous

or mercurous ions. Cobaltic ion is a more powerful oxidizing agent, but has not been studied because of its instability. The high positivity shown by gold at very low concentrations should probably be interpreted as an increase of the mean source potential,  $\bar{E}'_{st}$ , by oxidation of cytochrome *b*, cytochrome *c*, or both, either directly or through intermediate steps. Cytochrome *b* is the component of lowest redox potential (4), and accordingly the most readily oxidized. It has been shown that cupric ion, which is a much weaker oxidant than the auric ion, is capable of oxidizing the cytochromes (5).

The fact that the potentials are stabilized with all the heavy metals up to concentrations of  $10^{-4}$  M or higher would seem to indicate the reduction of the metallic ions by protective substances, possibly glutathione among others, until the concentration of metal becomes excessive. According to the experimental results, glutathione has a strong tendency to abolish the potentials, in comparison with NaCl as a rinsing agent. The thiol was applied in .01 M concentration in isotonic NaCl. Moreover the potential has been found to be minimal, when the tissues have previously been washed with glutathione. Evidently each of the metals is capable of forming complexes which may or may not affect the source potential. The auric complex, for example, tends to accept electrons from the cytochrome source, *b* or *c*; but the tissues are protected from the much more powerful oxidizing tendency of the free auric ion, no sharp increase in the observed potential occurring until the metal concentration reaches approximately  $10^{-4}$  M. In contrast with gold the ferric ion complex produces a slightly negative potential, but a sharp increase occurs at about the same concentration of metal,  $10^{-4}$  M. Mercury and copper at low concentrations are stabilized near the NaCl base line, but at concentrations in the neighborhood of  $10^{-5}$  M the potentials increase sharply, reaching a maximum at  $7.5 \times 10^{-4}$  M. Accordingly, in each case, breaks in the curves occur at concentrations where free oxidant ions occur in the tissues, and direct oxidation of the source cytochromes takes place. The fact that the sharp increase of potential occurs at a lower copper concentration than with iron or gold may be related to the strongly catalytic effect of copper ions on the oxidation of glutathione (6, 7). Pyrophosphate, which is a specific reagent for iron inactivation, does not inhibit the oxidation of glutathione in biological systems but activates it (8). Thus iron must be regarded as an inhibitor of glutathione oxidation, a fact which may be related to its tendency to yield negative potentials at low concentrations. Iron, as is evident from the data of table 1, gives a potential of only 67 mv., on the average, at a concentration of  $7.5 \times 10^{-4}$ ; and gives its highest potential at a concentration 10 times greater. The facts thus point to glutathione as an important stabilizer of the source cytochrome potential, and particularly indicate its importance in protecting against heavy metal oxidants. In addition to the direct oxidation of the source cytochrome component resulting in positive potentials, the results also yield evidence as to the importance of catalytic oxidation of glutathione, as with copper ions, and as to the importance of inhibition of glutathione oxidation, as with iron.

In the light of theoretical considerations the magnitudes of the potentials at the maximal levels appear to be significant. These are of the order of 150 mv. with copper and iron, 180 mv. with mercury and 230 mv. with gold. These figures compare with a difference of 300 mv. in the levels of cytochromes *b* and *c*, as estimated by Ball

(3). This value represents, according to the hypothesis, a theoretical upper limit of the effect, which would be obtained by shifting the cytochrome source potential from that of cytochrome *b* to that of cytochrome *c*. On the basis of the hypothesis, the results can be interpreted as follows. At concentrations of about  $10^{-4}$  M heavy metal oxidants have reached a lower limiting concentration above which they are able to oxidize cytochrome *b* directly, and inactivate it as an electron donor to the higher cytochromes. Cytochrome *c* remains available as an electron acceptor through succinic dehydrogenase and other enzymes and carriers. It has been shown that the oxidation of glutathione by cytochrome *c* is mediated probably by a copper enzyme (6). Succinic acid is oxidized by cytochrome *c*, the reaction being inhibited by either iron or copper inhibitors. These reactions are sufficient to account for a shift of source potential from the cytochrome *b* to the *c* level. Copper, for example, would activate such a process at a minimum of 3 points; the catalytic oxidation of glutathione, the direct oxidation of cytochrome *b*, and the coupling of hydrogen transport systems to cytochrome *c*. Iron would have similar effects, except for its inhibition of glutathione oxidation.

An additional property of the potential-concentration curves remains to be considered, namely the sharp maxima observed at about  $10^{-3}$  M concentration of auric or mercuric ions. These ions are more powerful oxidants than ferric or cupric ions. The mercuric-mercurous system has a standard redox potential of 0.901 volts, compared with 1.36 volts for the gold electrode and 0.81 volts for oxygen at *pH* 7.0<sup>3</sup>. Accordingly, either ion in the half oxidized state is capable of oxidizing cytochrome oxidase, which is itself oxidized by oxygen at the tensions prevailing in tissues. According to equations 1 and 2, oxidation of cytochrome oxidase increasing the terminal potential,  $\bar{E}_t$ , has the effect of decreasing the observed potential. The effect is opposite in sign to that of oxidizing the acceptor components *b* and *c*, which leads to a high source potential,  $\bar{E}_s$ , and to high positive observed potentials. The decrease of potential observed at high concentrations of gold or mercury is thus comparable to the negative potentials produced by iodine or hydrogen peroxide, which, apparently like oxygen, are electron acceptors from cytochrome oxidase.

#### SUMMARY

The effects of cupric, ferric, mercuric, auric, and cobaltous ions on the membrane potential across the synovialis have been determined in the knee joints of dogs. All the ions yield relatively small potentials up to concentrations of approximately  $10^{-4}$  or  $10^{-3}$  M, at which level there occur sharp positive increases. Only gold produces appreciably positive potentials in lower concentration ranges. On the basis of theoretical considerations the results are explained as oxidation of the electron acceptor components of the cytochrome system, either directly or indirectly. Protection of the tissue against heavy metal oxidants at low concentrations is interpreted as reduction and complex formation, with glutathione and other components. Individual characteristics of the metals are explained on the basis of this interpretation.

<sup>3</sup> Data from International Critical Tables.

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# ERYTHROCYTE AGE AND CHOLINESTERASE ACTIVITY

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**M**ARKED variations in the cholinesterase (ChE) activities of red blood cells have been noted in anemias. Sabine (1) and more recently Meyer *et al.* (2) have noted that low erythrocyte ChE activity occurs in patients with pernicious anemia in relapse. With the institution of adequate therapy the ChE activity of the red blood cells soon rises to above normal levels and then more slowly decreases to the normal range. Erythrocytes from patients with anemias due to increased blood destruction or loss of blood have been found usually to manifest an elevated ChE activity.

No adequate explanation has been offered to account for these interesting observations. This investigation was undertaken in an attempt to elicit the mechanisms responsible for such variations in erythrocyte ChE activity in anemias.

## EXPERIMENTAL

Male albino rats weighing 230 to 280 gm. were used for the study. Erythrocytes were obtained by bleeding from the tail into warmed heparinized saline or by bleeding directly from the aorta if the animal was to be discarded. Ether anesthesia did not alter the ChE activity of the red cells. The erythrocytes were washed twice with 5 volumes of normal saline.

ChE activity was measured with a Warburg manometer; 0.4 ml. of erythrocytes were hemolyzed in 3.0 ml. of 0.025 M sodium bicarbonate solution. In the sidearm was placed 0.2 ml. of acetylcholine bromide solution; the final concentration of acetylcholine was 0.015 M. The system was gassed with a mixture of 95 per cent N<sub>2</sub> and 5 per cent CO<sub>2</sub> for 10 minutes; the temperature was maintained at 37°. Starting 10 minutes after adding the acetylcholine from the sidearm, the CO<sub>2</sub> liberated during 40 minutes was measured. Production of CO<sub>2</sub> by spontaneous hydrolysis of acetylcholine was determined in the absence of red cells. The CO<sub>2</sub> production due to ChE activity was found to be linear during the 40 minute period. This is essentially the method of Ammon (3).

The ChE activities were not calculated as mean cell esterase activities since little variation in the mean corpuscular volumes of the erythrocytes was found.

Seven normal rats were subjected to repeated bleeding and the ChE activity of the red cells was determined at each bleeding; blood containing 1.8 to 2.2 ml. of erythrocytes was removed every 48 hours. Progressive increases in ChE activity occurred; after four bleedings the erythrocyte ChE activity was found to be at least

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100 per cent greater than originally present. Reticulocytoses of 50 to 80 per cent were observed. One rat (*rat C*) inadvertently continued to bleed from the tail during the third 48-hour period. When erythrocytes were next obtained, an increase in the ChE activity of 160 per cent was found, accompanied by 82 per cent reticulocytes. The rat died shortly after this bleeding.

Three of the rats after a rest period of 14 to 16 days were again bled. The erythrocyte ChE activities were found to have decreased and were only 5 to 15 per cent greater than originally present. Bleeding every 48 hours was continued. The erythrocyte ChE activities were again increased at rates similar to those previously noted. The results for 4 rats are shown in figure 1.

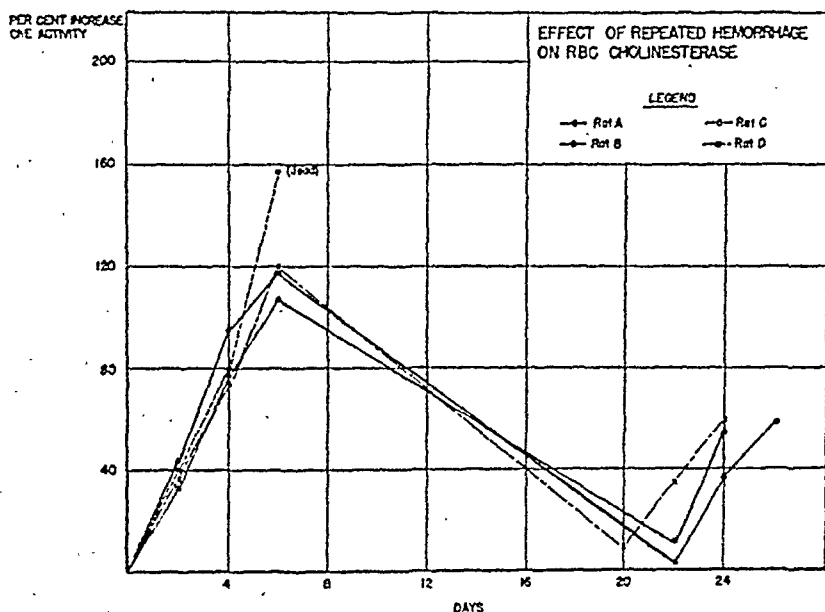


Fig. 1. INCREASE IN RAT ERYTHROCYTE CHOLINESTERASE ACTIVITY following repeated hemorrhage. At each of the points on the figure 1.8 to 2.2 ml. of erythrocytes were removed. Each point represents the percentage increase above the original RBC cholinesterase activity.

The observation that erythrocyte ChE activity is augmented following hemorrhage prompted attempts to obtain from the same specimens of blood erythrocyte samples differing in content of reticulocytes. It was found that this could be accomplished by centrifugation, taking advantage of the lower specific gravities of the reticulocytes. Heparinized blood from rats bled severely once or twice 48 to 72 hours before was diluted with an equal volume of normal saline and centrifuged in an angle centrifuge at 3000 revolutions per minute for 10 minutes. The upper half of the packed erythrocytes was then separated from the lower half by aspiration. The cells were washed with normal saline, reticulocyte counts were made, and the ChE activity of each of the red cell fractions was measured. The adhesiveness of the reticulocytes made counting in the reticulocyte-rich half somewhat difficult since they were often found in aggregates after being so centrifuged. An aliquot of blood whose reticulocyte distribution was not altered by such centrifugation was used to determine the erythrocyte ChE activity and reticulocyte level present before the reticulocyte distribution had been altered.

In the two experiments recorded in table 1 it can be seen that the red cells from the lower part of the centrifugates had low reticulocyte concentration (4 and 5%) and that the ChE activities were very similar to those found in the original normal erythrocytes at the time of the first bleeding. The red cells from the upper part of the centrifugate, however, possessed much higher ChE activities than were present in the lower or in the original erythrocytes. High reticulocyte concentrations accompanied the elevated activities. The ChE activities of the unfractionated erythrocytes were found to equal, within the limit of experimental error, the average of the ChE activities of the reticulocyte-rich and reticulocyte-poor fractions.

To attempt further to correlate increased erythrocyte ChE activity with recently formed erythrocytes, using the reticulocyte concentrations as an indicator of the

TABLE 1. CHOLINESTERASE ACTIVITY IN 'FRACTIONATED BLOOD'

RAT	ORIGINAL RBC ChE ACTIVITY		RBC ChE ACTIVITY 48 TO 60 HR. AFTER BLEEDING					
			Upper RBC's		Lower RBC's		Unfractionated RBC's	
	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$	% Retic.
11	99	3	215	52	96	5	160	32
12	89	2	194	43	88	4	147	22

TABLE 2. CHOLINESTERASE ACTIVITY AFTER ADMINISTRATION OF DFP

RAT	RBC ChE 24 HR. AFTER DFP		RBC ChE 48 TO 72 HR. AFTER BLEEDING			
			Lower RBC's		Upper RBC's	
	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$	% Retic.
1	18	2	18	2	80	20
2	41	2	31	3	157	34
3	12	3	38	9	124	29
4	15	3	24	4	258	73
5	24	4	43	5	91	19
6	23	3	54	6	171	30

amounts of new cells present, di-isopropyl fluorophosphate (DFP) was administered to rats to destroy the cholinesterase in the existing red cells and then hemorrhage was produced. DFP, recently dissolved in normal saline, was given intraperitoneally in one dose of 0.3 to 0.5 mg. Twenty-four hours later 2.0 to 3.5 ml. of erythrocytes were removed from the tail of each rat. The ChE activity of these cells was measured. With the doses of DFP used a reduction in ChE activity of 10 to 30 per cent of normal was obtained. Rats so treated were exsanguinated from the aorta 48 to 72 hours after hemorrhage. The blood was centrifuged in an angle centrifuge as described above and again marked differences in the reticulocyte concentrations between the upper and lower erythrocyte layers were obtained. Data are shown in table 2. The reticulocyte-poor lower layers of the centrifugates possessed ChE activities nearly equal to those of the erythrocytes obtained 24 hours after the administration of DFP. The ChE activities of the reticulocyte-rich erythrocyte fractions were again found to be much higher than those of the reticulocyte-poor fractions or of the original cells

obtained after DFP. The activity of each of the reticulocyte-rich fractions was proportional to the reticulocyte concentration within the limit of error in counting the reticulocytes.

Normal rat blood subjected to the same procedure of centrifugation also yielded erythrocyte fractions possessing distinct differences in ChE activity. The results with normal blood so treated are shown in table 3. While the reticulocyte concentrations were low in the upper portion of the centrifugates, the concentrations were distinctly higher than in the lower erythrocyte fractions, indicating a tendency of younger erythrocytes to collect in the upper part of the packed cells. The upper erythrocytes possessed ChE activities 50 to 70 per cent higher than the lower erythrocytes. Erythrocyte activities of aliquots of blood not so fractionated were equal to the average of the activities of the upper and lower erythrocyte fractions.

TABLE 3. CHOLINESTERASE ACTIVITY IN NORMAL 'FRACTIONATED' BLOOD

RAT	UPPER RBC'S		LOWER RBC'S		UNFRACTIONATED RBC'S
	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$	% Retic.	$\mu\text{l. CO}_2$
17	135	4.1	55	1.2	96
19	203	5.1	91	1.8	140
20	182	5.6	88	1.6	131

#### DISCUSSION

The data presented show that in the rat, concomitant with the production of new erythrocytes following hemorrhage, marked increases in red cell ChE activity occur. These augmentations are not permanent, for ChE activities were noted to have decreased to near the original levels within 14 to 16 days after the last bleeding. That the ChE activity is greatest in young erythrocytes is shown by the use of centrifugation to alter the distribution of young erythrocytes in the same blood specimen, the blood being either normal or that obtained following hemorrhage. Using DFP to destroy nearly all the ChE activity of circulating erythrocytes and then producing hemorrhage, centrifugation yielded reticulocyte-poor fractions which contained few reticulocytes and which possessed ChE activities not much different from those of the erythrocytes originally present after DFP. The reticulocyte-rich fractions, however, showed greatly augmented ChE activities; the increases were proportional to the reticulocyte concentrations.

Observations of the effect of hemorrhage and of the effect of altering young red cell distribution by centrifugation on the erythrocyte ChE activities of a small group of rabbits were also made. The results were very similar to those found in rats. Data obtained from the rabbit experiments have not been included in this report.

Dr. Jean Sabine in a personal communication states that blood from a pernicious anemic during active reticulocyte formation, when treated as described above, yields reticulocyte-rich erythrocyte fractions containing much higher ChE activities than the reticulocyte-poor fractions.

Neither the rate of decrease of ChE activity, as the erythrocyte becomes older, nor the cause of such a decrease can be determined from these experiments. The

data suggest, however, that at least one cause of the variations in erythrocyte ChE activity found in anemias is the lower ChE activity of older red cells. Thus in pernicious anemia in relapse, where a majority of the erythrocytes are older cells, the low ChE activity which is found would be anticipated. The increase in ChE activity to above normal levels following therapy as many new cells are released into the circulation, shifting the cell population to predominantly younger erythrocytes, and the gradual decline of ChE activity to normal as a normal balance of younger and older cells is reached, also can be explained by the difference in ChE activity of younger and older erythrocytes.

In anemias due to increased blood destruction or blood loss the elevated erythrocyte ChE activities can be accounted for by the shift in the cell population to younger red cells. The observation of Meyer (4) that some cases of severe hemolytic anemia do not show elevations of erythrocyte ChE activity cannot be explained, however, by this mechanism.

#### SUMMARY

Repeated hemorrhage in rats produces progressive increases in erythrocyte cholinesterase activity. Upon cessation of hemorrhage the ChE activity declines to normal levels. When centrifuged, rat erythrocytes distribute so that younger red cells are found in the upper part of the centrifugate. These red cells possess much higher cholinesterase activities than erythrocytes found in the lower regions of the packed cells. A possible mechanism explaining the variations of erythrocyte cholinesterase activity in anemias is offered.

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# INFLUENCE OF SNAKE VENOM ON MAMMALIAN ERYTHROCYTES IN VITRO

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FLEXNER and Noguchi (1) reported that snake venom would bring about agglutination of washed erythrocytes, but that serum or plasma containing complement was necessary for hemolysis. Kellaway and Williams (2), on the other hand, observed that the venom of certain Australian snakes and the Indian cobra would hemolyze the washed erythrocytes of certain species of mammals and that homologous serum or plasma mixed with the cells of many of these species would actually inhibit hemolysis. With the cells of certain other species, however, serum activated hemolysis. In view of this disagreement in the results of these American and Australian workers additional investigation of the problem seemed desirable.

## MATERIALS AND METHODS

*Venoms.* These were from the water mocassin (*Agkistrodon piscivorus*), copperhead (*Agkistrodon mokasen*), and rattlesnake (*Crotalus adamanteus*) and were obtained from Ross Allen's Reptile Institute in dry powdered form. The water mocassin and copperhead venoms had been extracted 3 years before use, and the rattlesnake venom one and one-half years before use. Ross Allen (3) states that he 'milks' each snake only once after it has been captured. This venom is dried soon after extraction and kept in cold storage until it is shipped.

Two per cent venom solutions were prepared by dissolving one part of dried venom by weight in 49 parts of 0.85 per cent sodium chloride solution by volume. When not used immediately, these solutions were stored at  $-20^{\circ}\text{C}$ .

*Bloods.* These were obtained from man, dog, sheep and rabbit by means of venipuncture. Two to 3 ml. of whole blood were placed in 100 ml. of 0.85 per cent saline solution, and the remainder placed in a test tube and allowed to clot. Serum was obtained by centrifuging the clotted blood and withdrawing the supernatant liquid. When not used immediately, the serum was stored at  $-20^{\circ}\text{C}$ .

The saline suspensions of erythrocytes were washed six times in 50 volumes of normal saline and were finally made up as one or 2 per cent suspensions by volume. When not used immediately, these cells were stored at  $4-6^{\circ}\text{C}$ . for not longer than one week. The canine erythrocyte suspensions were not kept after 48 hours from the time of extraction because they underwent spontaneous hemolysis soon after that time.

## EXPERIMENTAL PROCEDURE AND RESULTS

*Experiment 1.* Two tubes of each of the several dilutions of water mocassin, copperhead, and rattlesnake venom were prepared in 0.4 ml. volume in concentrations

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TABLE 2. REACTION OF WATER MOCCASIN VENOM ON CELLS OF DIFFERENT SPECIES OF MAMMALS WITH OR WITHOUT HOMOLOGOUS SERUM

A. Readings after 3 hours at 37° C.

WATER MOCCASIN VENOM		Initial Dilutions of Venom									
		3200	6400	12,800	25,000	50,000	100,000	200,000	400,000	800,000	Control
Dog RBC—Saline	a	+++	+++	+++	+	—	—	—	—	—	—
	b	+	+	—	—	—	—	—	—	—	—
Dog RBC—Serum	a	+++	+++	+++	+++	+++	+++	++	+	—	—
	b	—	—	—	—	—	—	—	—	—	—

B. Readings after 24 hours at 37° C.

		200	400	800	1600	3200	6400	12,800	25,000	50,000	Control
Human RBC—Saline	a			+++	+++	+++	+++	+++	+++	+++	—
	b			—	—	—	—	—	—	—	—
Human RBC—Serum	a			+++	+++	++	++	++	++	—	—
	b			+	+	+	+	+	+	—	—
Rabbit RBC—Saline	a			+++	+++	+++	+++	+++	+++	++	—
	b			—	—	—	—	—	—	—	—
Rabbit RBC—Serum	a			+++	+++	+++	+++	++	—	—	—
	b			—	—	+	+	+	—	—	—
Sheep RBC—Saline	a	—	—	—	—	—	—	—	—	—	—
	b	—	—	—	—	—	—	—	—	—	—
Sheep RBC—Serum	a	?	?	?	?	—	—	—	—	—	—
	b	XXXX	XXXX	XXXX	XXXX	XXXX	—	—	—	—	—

X = Increased cell volume. Note: Readings designated as in table 1.

indicated in table 1. To one series of each venom dilution 0.1 ml. of human serum was added, to the other series of each, 0.1 ml. normal saline was added. Four-tenths ml. of the washed human erythrocyte suspension was placed in each tube. Control tubes for each series were set up containing all of the components of the other tubes, except that 0.4 ml. normal saline was used in place of the venom dilution.

Results outlined in table 1 show that venom from the three species of snakes brought about hemolysis of *washed* human erythrocytes, and human serum actually inhibited this hemolysis. Water moccasin and copperhead venoms brought about agglutination of erythrocytes after 3 hours, but very little hemolysis. However, every instance of agglutination of washed erythrocytes at 3 hours was followed by almost complete hemolysis at 24 hours. Agglutination and hemolysis of washed erythrocytes by the rattlesnake venom was not as great as with the water moccasin and copperhead venoms. The presence of human serum appeared to inhibit agglutination of human erythrocytes to the same degree that it inhibited hemolysis. However, though hemolysis was inhibited by serum after 24 hours, the presence of serum after 3 hours appeared to hasten hemolysis while at the same time inhibiting agglutination.

Hemolytic activity at 3 hours with the 1 to 50 dilutions of water moccasin and copperhead venoms was not as great as with some of the higher dilutions. This phenomenon of inhibition by venom excess is a common observation.

*Experiment 2.* Water moccasin venom, which was the most potent of the three on human cells, was then tested with the cells of the dog, rabbit and sheep with and without homologous serum. Human cells were run simultaneously for comparison. The procedure was identical with that carried out in *experiment 1*.

It could not be ascertained that actual lysis of sheep cells ever occurred. However, there was an apparent increase in cell volume as indicated in table 2, and this increase in cell volume is, according to Essex and Markowitz (4), a step toward hemolysis. Therefore, the activity of water moccasin venom on dog and sheep erythrocytes is enhanced by the presence of homologous serum, whereas with rabbit and human cells, serum inhibits hemolysis. Rabbit cells were very similar to human cells in their susceptibility to water moccasin venom. Phosphate buffered saline (pH 7.2) was used for the dog erythrocytes since they appeared more stable in this medium than with unbuffered saline. Readings on dog cells were not taken after 3 hours because of the instability of these cells in saline solutions.

*Experiment 3.* This experiment was performed to compare the effects of the rattlesnake venom on washed dog cells and on dog cells with fresh dog serum, inactivated dog serum (heated at 56° C. for 45 minutes), and rabbit serum. Heated dog serum was used because heat inactivates complement. Rabbit serum was used to find out if heterologous serum acts the same as homologous serum on venom hemolysis. The procedure was the same as for the preceding experiment.

The results in table 3 show that fresh dog serum had greater activating powers than either inactivated dog serum or rabbit serum. However, assuming that complement is inactivated by heating it at 56° C. for 45 minutes, then serum complement is not necessary for the activating properties of dog serum. Substances in heterologous serum also appear to have activating properties. Rabbit serum inhibited the action

of water mocassin venom on rabbit cells, whereas it activated the rattlesnake venom hemolysis of dog cells. Dog erythrocytes with serum were very much more susceptible to the action of the rattlesnake venom than human cells, and whereas human serum inhibited hemolysis of human red cells by the venom of this species, serum appeared to be necessary for hemolysis of dog cells.

TABLE 3. REACTION OF RATTLESNAKE VENOM ON DOG ERYTHROCYTES WITH AND WITHOUT VARIOUS TYPES OF SERUM

		<i>Readings after 3 hours at 37° C.</i>								
DOG EBC		Initial Dilutions of Venom								
		10,000	20,000	40,000	80,000	160,000	320,000	640,000	1,280,000	Control
Saline	a	—	—	—	—	—	—	—	—	—
	b	—	—	—	—	—	—	—	—	—
Fresh Dog Serum	a	++++	++++	++++	++++	++++	++++	++++	++	—
	b	—	—	—	—	—	—	—	—	—
Inactivated Dog Serum	a	++++	++++	+++	++	++	++	+	±	—
	b	—	—	—	—	—	—	—	—	—
Rabbit Serum	a	++	++	+	+	+	±	—	—	—
	b	—	—	—	—	—	—	—	—	—

Note: Readings designated as in table 1.

#### DISCUSSION

After complete lysis of human, dog and rabbit cells had taken place, the color of the solution often changed from red to straw. Sheep cells after swelling appeared yellow in suspension and brown after settling together in the bottom of the tube. This phenomenon might be explained by Dunn's report of the change of hemoglobin to methemoglobin by snake venoms (10).

Flexner and Noguchi (1) failed to record how long their observations were made after the venom was mixed with the cells. Experiments with human, sheep and rabbit cells (tables 1 and 2) show that maximum hemolysis requires 24 hours and that 3-hour readings alone are often misleading. Dog serum heated at 56° C. for 45 minutes and sheep serum 6 months old retained much of their activating properties, indicating that when serum does serve as an activator it does not depend entirely upon complement. Furthermore human serum heated at 56° C. for 45 minutes retains its inhibitory properties.

The experiments recorded in this article are essentially a confirmation of the work of Kellaway and Williams (2). These workers concluded that venom must unite with lecithin before hemolysis can occur. Cells which have free lecithin in their stroma capable of combining with the enzymes of venom can be hemolyzed without an activator; cells which do not have free lecithin in their stroma require an

activator such as serum or plasma. The characteristic activating or inhibiting properties of serum in their experiments were dependent upon the whole serum protein-lipoid complex, rather than any fraction of it. Kellaway (5) in a later report said that venom reacted with lecithin by splitting off one molecule of oleic acid. Lysollecithin is probably responsible for prehemolytic swelling of the erythrocytes and is inhibited by egg albumen. Delezenne (6) performed experiments showing the enzymatic nature of the action of venom on erythrocytes using horse serum as an intermediary substance.

The experiments reported herewith also essentially confirm the work of Essex and Markowitz (4) in which these workers observed that serum or plasma was necessary for rattlesnake venom hemolysis of dog cells<sup>1</sup>. The present work, however, shows that results obtained on dog cells (*in vitro*) do not apply to human cells. The variations between these two species have been pointed out previously. Essex and Markowitz (4) made the very interesting observation that oxalate hampers the activating properties of dog plasma. Therefore, in venom hemolysis, there is an enzyme system involving proteins (venom), phospholipids and possibly calcium. Schrek (7) has also reported that mocassin venom has the capacity of agglutinating and lysing lymphocytes, but no effect on the viability or motility of the polymorphonuclear cells. Lysollecithin in addition to its hemolytic action also causes liberation of histamine from the perfused lungs, decreased excitability of the isolated jejunum, contraction of the guinea pig uterus, liberation of protein and pigments from the perfused monkey liver, and many other pharmacological phenomena (8). Zeller (9) in a very comprehensive report on snake venom states that "the lecithinase of snake venom renders capillaries more fragile by attacking the lipid layer of endothelium and thereby aids proteinase in bringing about internal hemorrhages." The actions of all the enzymes of snake venom are dispersed *in vivo* by the spreading action of hyaluronidase accompanied by proinvasion I which aids in counteracting normal mechanisms of defense of hyaluronic acid.

#### SUMMARY

Water mocassin and copperhead venoms bring about agglutination and subsequent hemolysis of washed human erythrocytes. The venom of the rattlesnake, *C. adamanteus*, also hemolyzes washed human erythrocytes, but not to the same degree as occurs with water mocassin and copperhead venoms. Human serum inhibits the hemolytic activity of the 3 venoms on human cells. Serum of the dog and sheep activates the action of water mocassin venom on their homologous erythrocytes, whereas serum of the rabbit and man acts as an inhibitor. Dog cells in the presence of serum are much more susceptible to the action of rattlesnake venom than are human cells. Fresh dog serum, heated dog serum and rabbit serum will activate rattlesnake venom hemolysis of dog cells.

This work was done as a result of suggestions by Dr. H. S. Mayerson and correspondence with Dr. Hiram Essex. Dr. Ernest Carroll Faust and Dr. Albert Zeller have contributed other useful suggestions and criticisms. The experiments were performed in the laboratory of bacteriology under the technical supervision of Dr. M. F. Shaffer and Dr. Paul Donaldson.

<sup>1</sup> Hemolytic activity in their experiments did not proceed further than a pronounced swelling of the erythrocytes.

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# PLATELETS AS FOCI IN THE COAGULATION OF BLOOD

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**N**UMEROUS morphologic observations, especially those of Fonio and Schwendener (1), suggest that the platelets have an important, possibly an initiating role, in the coagulation of blood. In order to retard the clotting process enough to permit accurate observation, Fonio and Schwendener employed various anticoagulants and low temperatures. Tocantins (2), on the other hand, studied the clotting of hemophilic plasma and observed that fibrin threads formed independent of the platelets. Although platelets do not contain an appreciable amount of prothrombin, recent work (3-6) indicates that there is a material associated with them which facilitates the conversion of prothrombin to thrombin. Normal human plasma clots slowly enough in silicone-coated glass to permit observation of the process. It is such observations which we wish to report.

We also compared platelets to plasma in content of what might be termed 'prothrombin conversion factors' and shall include the results of our comparison.

## MICROSCOPIC OBSERVATIONS

Normal human blood was drawn by the silicone technic of Jaques and co-workers (7). The plasma was separated by centrifuging the blood in silicone-coated tubes; a small drop of plasma was transferred by means of a silicone-coated pipet to a similarly treated slide and to a coverglass preparation which was then sealed with petrolatum. The clotting of the plasma was observed by means of the phase-contrast microscope, and the structure of the platelet and fibrin was sharply visualized. Still photographs of the free platelets could not be made because of their constant brownian movement.

The platelets in the silicone preparations differed from those seen in oxalated plasma exposed to glass in two respects: 1) agglutination was absent and 2) there were initially few cytoplasmic processes. Processes similar to those illustrated by Fonio and Schwendener did appear somewhat rapidly, however, and were well developed long before any fibrin had formed. Since the platelets frequently presented themselves in profile it could be seen that all of the processes arose from the rim of the platelet disk. The processes had such delicately tapered ends that their length could not be determined exactly, but it was often at least 5 times the diameter of the platelet and may well have been much greater. The processes were in continuous motion, apparently in response to the brownian movement of the platelets, so that it seemed reasonable that their very delicate ends might break off, but this could not be actually seen.

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Fibrin first became visible about an hour after the blood had been drawn. Despite the slowness of the coagulation, the fibrin appeared rather suddenly as typical refractile needles which shot through the plasma in all directions. These first fibrin needles were relatively delicate and few in number. They rapidly became connected with platelets in their vicinity. As soon as one of the vibrating platelets was contacted by the fibrin it ceased its brownian movement and its processes could no longer be seen. Active migration of platelets to the fibrin could not be discerned and groups of platelets did not form. As additional fibrin threads formed they also became attached to platelets and as the fibrin threads grew they tended to become thicker at the point of attachment. Thus in a relatively short time all of the platelets became firmly fixed in the fibrin network which then presented the structure shown in

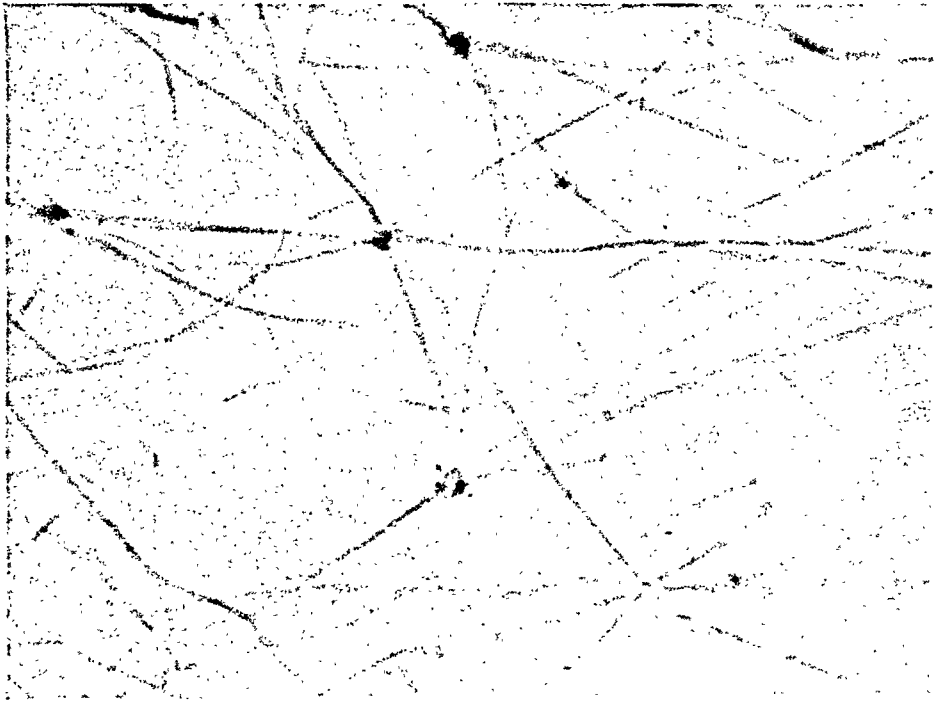


Fig. 1. STRUCTURE OF FIBRIN NET ( $\times 950$ ).

figure 1. Since fibrin threads appear to radiate from the platelets to which they are strongly attached and since the attachments of the fibrin appear to replace the platelet processes, the impression may readily be gained that the processes might be precursors of the fibrin threads. A connection however, was never seen between a platelet and a fibrin thread at the very moment of its formation. Thus it seems probable that, under the conditions of these observations, the first fibrin threads which are formed are free in the plasma and shortly thereafter become attached to the platelets. Whether or not the broken processes of platelets may be sites of origin of fibrin threads remains an interesting possibility concerning which we could not find evidence. The formation of the fibrin network occurred without any evident change in the number of platelets or any major change in their structure except for the loss of their processes.

## TESTS FOR PROTHROMBIN CONVERSION FACTORS

*Methods.* One-stage and two-stage assays of prothrombin were performed as previously described (8, 9). Crude platelet extract, aged plasma and plasma inactivated by zymin and ammonia were also prepared as in previous reports (1, 10). It should be noted that the three types of inactivated plasmas cannot be regarded as standardized reagents. They are merely specimens of plasma which have been treated in such a manner that the prothrombin clotting time has been markedly prolonged. This time can be restored to normal or nearly normal by the addition of serum, which does not contain either prothrombin or fibrinogen. Plasma inactivated by zymin and mixed with either aged plasma or ammonia-inactivated plasma shows a much shorter prothrombin time than that of either of the inactivated plasmas alone. All three inactivated plasmas give normal yields of thrombin by the two-stage method, provided serum is added. These inactivated plasmas may be

None	66		
Mixed with patient's fresh plasma 50:50	67		
0.1 cc. saline added		67	76
0.1 cc. patient's fresh plasma added		36	65

<sup>1</sup> Possible deficiency of 'labile factor' of Quick. Two-stage prothrombin level, 275 U/cc. Quick prothrombin time, 66 sec.

used to test for substances capable of shortening their prothrombin times, in a manner analogous to the use of specifically inactivated serums to test for components of complement. In their preparation, plasma inactivated by zymin or ammonia is diluted with an equal volume of imidazole buffer. As is pointed out in tables 1, 2 and 3, in some instances equal parts of the plasma to be tested and of inactivated plasma were mixed, as was done by Quick, although in most instances the material to be tested was simply added to the inactivated plasma, thereby increasing the volume of the clotting system from 0.3 cc. to 0.4 cc. In either case and for a number of reasons, the various conditions which may affect clotting time are less ideally arranged than in the standard Quick prothrombin time and the results for the present should be regarded as qualitative rather than quantitative.

*Results.* Merely for the purposes of this presentation, a prothrombin conversion factor is defined as "a material which favors the conversion of prothrombin to thrombin in the presence of tissue thromboplastin but which is not itself transformed into thrombin." Such a definition might possibly include the following factors reported in the literature: prothrombin A, Quick, 1943 (11); plasma accelerator, Fantl and Nance, 1946 (12); factor V, Owren, 1947 (13); Ac-globulin, Ware, Guest and Seegers, 1947 (14); labile factor, Quick, 1947 (15) (new name applied to prothrombin A, Quick, 1943); prothrombin A, Quick, 1947 (15) (a factor differing from

prothrombin A, Quick, 1943). Quick (15, 16) has stressed his important observation that clinical hypoprothrombinemia as detected by his one-stage assay can be divided into three distinct types; namely, deficiencies of prothrombin B (probably synonymous with the term 'prothrombin' as used by various authors), of the labile factor, and of prothrombin A. The first type mentioned is the common one and may readily be recognized by the two-stage method. Rarely, however, blood is observed with very abnormal prothrombin times and normal two-stage prothrombin levels.

TABLE 2. TESTS FOR PROTHROMBIN CONVERSION FACTORS IN CASE OF PATIENT WITH SEVERE BLEEDING TENDENCY OF UNKNOWN ETIOLOGY BUT NOT CONGENITAL<sup>1</sup>

TREATMENT OF INACTIVATED PLASMA	PROTHROMBIN CLOTTING TIME, IN SEC.		
	Aged plasma	Zymin-inactivated plasma	Ammonia-inactivated plasma
None.....	120		
Mixed with patient's fresh plasma 50:50.....	29		
0.1 cc. saline added.....		77	125
0.1 cc. patient's fresh plasma added.....		75	38

<sup>1</sup> Possible deficiency of 'prothrombin A, Quick, 1947.' Two-stage prothrombin level, 275 U/cc. Quick prothrombin time, 51 sec.

TABLE 3. RESTORATION OF COAGULABILITY TO INACTIVATED PLASMAS BY MEANS OF PLATELETS AND PLASMA<sup>1</sup>

MATERIAL ADDED TO INACTIVATED PLASMA	PROTHROMBIN CLOTTING TIME, IN SEC.		
	Aged plasma	Zymin-inactivated plasma	Ammonia-inactivated plasma
Saline.....	165	202	140
Platelet extract.....	14	96	22
Platelet extract diluted 1:10.....	22		24
Fresh plasma diluted 1:10.....	40	53	42
Serum.....		17	

<sup>1</sup> One-stage assays by Quick technic with the addition of 0.1 cc. of designated material to plasma, making total volume of system 0.4 cc. One cc. of platelet extract, made from the platelet sediment of 16 cc. of plasma with prothrombin content of 340 U/cc., contained less than 5 U of prothrombin. Platelet extract compared to the plasma from which it was made.

These appear to be due to deficiencies of prothrombin conversion factors, and we have also observed two types of such deficiency. In one type (table 1) a shortening of the prothrombin time results when the patient's plasma is mixed with zymin-inactivated plasma but not when it is mixed with aged or ammonia-inactivated plasma, while with the other type (table 2) just the reverse is true. It cannot be stated with certainty that these two types correspond to those observed by Quick, especially since the clinical features of the cases (to be reported elsewhere) are entirely different. Furthermore, since we regard our tests as only qualitative at present, it should not be claimed that these are examples of deficiencies of purely one factor. However, the observation that two types of deficiency occur spontaneously may be regarded as evidence that the difference previously observed between zymin-inactivated plasma and aged or ammonia-inactivated plasma may correspond to at least two prothrombin

conversion factors which exist naturally. Hence, it seemed appropriate to test platelets for prothrombin conversion factors in the same manner as was done with the deficient plasmas.

When platelet extract was added to the three types of inactivated plasma (table 3), it was found to have considerably more activity in restoring aged or ammonia-inactivated plasma than a comparable volume of the plasma from which it was made. On the other hand, it had very little activity in restoring zymine-inactivated plasma, perhaps no more than could be explained on the basis of the amount of normal plasma present in the platelet extract.

#### COMMENT

It appears from the foregoing experiments that part but not all of a complex of factors which favor conversion of prothrombin to thrombin is concentrated in or on the platelet. Ware, Fahey and Seegers (4), in experiments with purified coagulation factors, have found interesting qualitative as well as quantitative differences between prothrombin conversion factors found in platelets, in plasma and in serum. As was the case in Tocantins' study of hemophilic platelets, we were unable to find any morphologic evidence of initiation of fibrin formation by normal platelets, although conditions were such that one might hope to observe this phenomenon. Our observations would be consistent with the concept that platelets, by their peculiar morphologic and biochemical make-up, permit the rapid growth of a strong fibrin net in the presence of minimal concentrations of thrombin and thromboplastin, the two coagulation factors which, in excess, directly endanger life.

#### SUMMARY

Normal platelets were not seen to initiate fibrin formation in normal plasma, enclosed in silicone-coated surfaces. The platelets did serve as foci of formation of the fibrin network. Part but not all of a complex of factors which favor rapid conversion of prothrombin to thrombin is concentrated in or on the platelet.

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# A STUDY OF PULMONARY VENOUS AND ARTERIAL PRESSURES AND OTHER VARIABLES IN THE ANESTHETIZED DOG BY FLEXIBLE CATHETER TECHNIQUES

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IN THIS paper we shall describe measurements of pulmonary artery and pulmonary vein pressures and cardiac outputs in the dog with a closed chest. The values obtained in 64 nembutal anesthetized dogs will be presented, correlated with systemic arterial pressures and other variables. Many previous observations have been made on lesser circulation pressures and the literature will not be reviewed at this time. Among others, Hamilton, Woodbury and Vogt (1) have measured the pulmonary arterial and venous pressures relative to the intrathoracic pressure. They employed a differential manometer in trained unanesthetized dogs in which modified London cannulae had been placed at a previous operative procedure. With the recent advent of right heart and pulmonary arterial catheterization, as introduced by Forsmann (2) and employed by Cournand *et al.* (3-7); a promising new tool for investigating the lesser circulation was supplied. Hellemis, Haynes, Dexter and Kinney (8, 9) have catheterized the pulmonary artery and vein in the dog.

## METHODS

Mongrel dogs weighing more than 12 kg. were injected intravenously with sodium pentobarbital, 33 mg/kg. supplemented later as indicated. The right carotid artery, right jugular vein and trachea were isolated. After the vein was ligated, a no. 10 whistle-tipped radio-opaque ureteral catheter was inserted about 20 cm. in a central direction and tied in place. The catheters had a single terminal orifice in addition to two side openings within  $2\frac{1}{2}$  cm. of the tip. The total length of the catheters was 70 cm. A no. 15 needle was fitted into the end of the catheter and this in turn to one arm of a glass T tube by a metal adapter. Incorporated into a second arm of the T tube was a glass stopcock through which could flow a 5 per cent solution of glucose from a Murphy drip and flask. To prevent blood from clotting in the catheter and plugging its lumen, the glucose solution was permitted to drip at a rate of about 15 to 20 drops per minute. Heparinization was found to be unnecessary as long as one did not interrupt the flow of glucose solution longer than a minute at a time for pressure recordings. The carotid artery was ligated and catheterized in a similar manner. The perfusion fluid was delivered under 200 mm. Hg pressure.

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The catheter in the right jugular vein was then passed to the pulmonary artery under direct fluoroscopic vision. It was noted that in the dog the lateral fluoroscopic image of the catheter in the pulmonary artery was almost identical with the appearance of the catheter coiled once in the right atrium with the tip in the azygos vein. A test of the pressures encountered distinguished between the two positions. Pressures from 10 to 20 mm. Hg indicated that the tip was in the pulmonary artery, whereas pressures around zero pointed to the catheter's being in the azygos vein. Placing the catheter in the pulmonary vein was a more difficult procedure. First, the carotid artery tended to go into spasm making it difficult, especially in small dogs, to advance the catheter. This was overcome by selecting large animals and by lubricating the outside of the catheter with small quantities of mineral oil. Second, since the catheter had to advance against the flow of arterial blood, there was a tendency for it to double back on itself, at times lodging tightly in this manner in the carotid artery. This occasionally defeated all attempts either to advance or withdraw the catheter. The catheter was advanced down the right carotid into the brachiocephalic artery and the aorta. In order to facilitate its use a 30° curve was placed in the distal 5 cm. of the catheter. It could then be turned so as to traverse the arch of the aorta toward the heart. The catheter usually slipped past the aortic valves with relative ease after a few moments of manipulation. Once in the left ventricle the catheter took one of two courses through the mitral valve. The preferred course, but the one more difficult to achieve, was to have the catheter tip turn dorsalward at a right angle (fig. 1) and pass through the mitral valve without making a loop in the left ventricle. In most instances, however, the catheter did loop in the ventricle (fig. 2) before entering the atrium. Both courses are acceptable though the latter unnecessarily increases catheter length, in addition to placing a larger foreign body in the heart. Furthermore, a looped catheter whipping in the left ventricle increases the possibility of pressure-recording artifacts. After passing the mitral valve the catheter usually dropped readily into a pulmonary vein. The catheter most often entered the vein draining the left lower lobe, less often the right lower or middle lobes but never an upper lobe. When the vein was reached, the catheter was adjusted so that it extended about 2 cm. into its lumen. A ligature tied about the catheters 1 cm. before their exit from the carotid artery and jugular vein held them in position.

It must be emphasized that there is a significant mortality from left heart catheterization. Seven deaths occurred during passage of the left catheter in 71 trials, while none occurred from passage of the right catheter. Three of the deaths occurred when the catheter doubled back and lodged tightly in the carotid artery. Attempts at moving it resulted in shearing of the artery from the aorta. One death occurred when the catheter tip perforated the left ventricle. Another occurred when the tip perforated the aorta just above the valves and entered the pericardial space. One animal died suddenly while the catheter was being manipulated in the left ventricle. No lesions could be found at necropsy. It was thought the death was probably due to ventricular fibrillation, since it is not unusual for extrasystoles to occur during catheterization of either side of the heart. The seventh death occurred when due to error air was forced into the heart under 200 mm. Hg pres-

sure. The proportion of deaths, as well as the length of time required to pass the catheter, decreased with increasing experience. Post-mortem evidence of valvular damage was observed in none of the animals. Small subendocardial hemorrhages were occasionally noted in the left ventricle. However, this finding is frequently seen in animals not subjected to left heart catheterization (10).

Intrathoracic pressure was measured by inserting an abdominal trocar and cannula into the pleural space between the right 5th and 6th ribs at the apex of the chest. The cannula had an internal diameter of 4 mm. and 2 longitudinal slits which extended  $1\frac{1}{2}$  cm. from the tip. The trocar was removed and about 25 cc. of air injected to produce a small pneumothorax at the position of the cannula.



Fig. 1. CATHETERS IN A PULMONARY ARTERY and a pulmonary vein. Upper looped catheter in pulmonary artery shows a double shadow with ventricular contraction. Lower catheter in a pulmonary vein without first looping in left ventricle.

Pulmonary artery and vein pressures were measured with a standard 0-6 p.s.i. resistance wire pressure transmitter (Statham strain gauge)<sup>1</sup> (11-13) connected to a type A oscillograph galvanometer.<sup>2</sup> Intrathoracic pressure was measured with a 0-1 p.s.i. pressure transmitter connected to a type B oscillograph galvanometer. The integrated mean vascular pressures, referable to the intrathoracic pressure were calculated from the photographic record with the use of a compensating polar planimeter. Calibration of the apparatus showed a straight line relation between pressure and deflection over the ranges employed and the calibration did not change over 11 months of use. Though the natural frequencies of the strain gauges and galvanometers used are above 100 cycles per second, it should be noted

<sup>1</sup> Statham Laboratories, 9328 Santa Monica Blvd., Beverly Hills, Calif.

<sup>2</sup> Heiland Research Corporation, 130 E. 5th Ave., Denver, Colorado.

that the overall frequency of the entire system was about 15 c.p.s. That the mean pressure values were not vitiated by artifacts is indicated by experiments in which pressures were recorded with a mercury manometer and a Hamilton differential manometer in addition to the strain gauge. In each case the mean pressures checked very closely. The Hamilton manometer was not used routinely because of inherently greater practical difficulties. Peripheral arterial pressure was measured with a mercury manometer by cannulating the left femoral artery. Peripheral venous pressure was measured in the left jugular vein with a sodium citrate manometer.

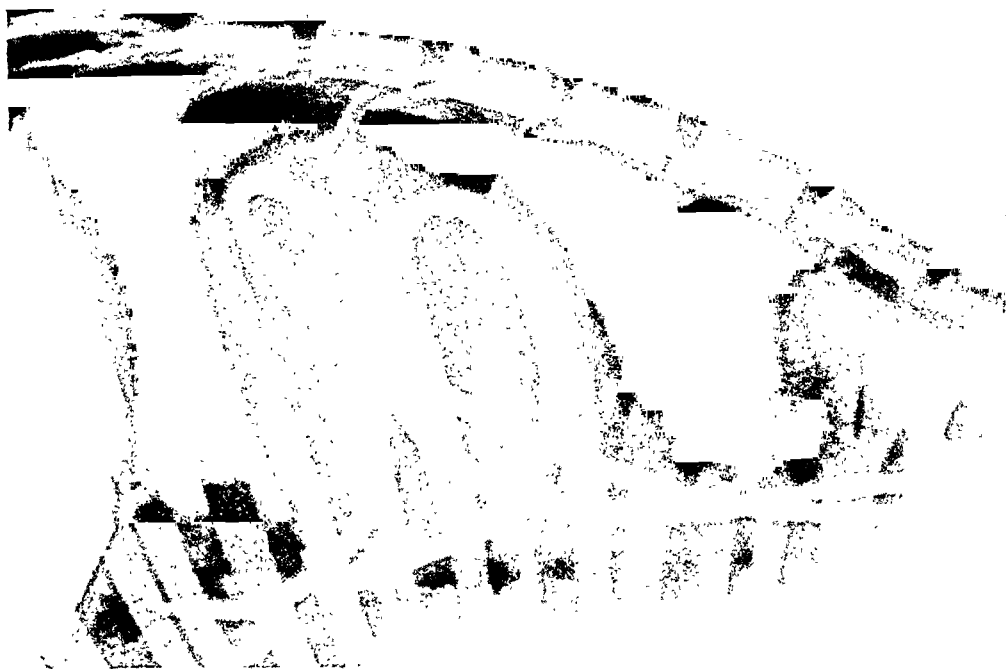


Fig. 2. CAUDAL CATHETER TIP IN A PULMONARY VEIN after first looping in left ventricle. Cephalad catheter tip in a pulmonary artery. Both catheters show double shadow with cardiac contraction. Catheters were withdrawn to a position nearer the heart during recording of pressures.

from the right femoral artery. Using a closed system spirometer connected to the tracheal cannula, oxygen consumption was measured over an eight minute period. Oxygen content of the blood samples was determined by the Van Slyke manometric method (14). Using Rubner's constant in Meeh's formula,  $11.2 \sqrt{(\text{wt. in gm.})^2}$ , to calculate body surface area, cardiac output was expressed as cardiac index.

#### RESULTS

Cardiac output was estimated by the direct Fick method. Mixed venous blood was obtained from the catheter in the pulmonary artery. Arterial blood was taken

A statistical treatment of the results of observations on pressures and cardiac outputs in 64 dogs is presented in table 1. The values shown are those obtained immediately after completing the preparation of the animal for study. The data are segregated in this table in relation to the existing systemic arterial pressure.

The significance of differences between means has been calculated by application of the Fisher *t* test. The probabilities of the differences between means occurring by random chance are shown in a footnote to the table.

It will be seen that the average integrated mean pulmonary arterial pressure is significantly higher in nembutalized dogs with systemic arterial pressures above 150 mm. Hg than in those below 100 mm. Hg. The value at intermediate arterial pressures is, as would be predicted, intermediate, but the probability calculation shows that the differences are at the borderline of significance,  $P = 0.05$ .

Correlated with these observations are those on pulmonary venous pressure, where highly significant positive correlations are found between the low and intermediate as well as the low and high systemic blood pressure groups. No significant

TABLE I. RELATION OF PULMONARY PRESSURES AND CARDIAC INDICES TO PERIPHERAL ARTERIAL BLOOD PRESSURE<sup>1</sup>

MEAN SYSTEMIC ARTERIAL PRESSURE	MEAN PULMONARY ARTERY BLOOD PRESSURE <sup>2</sup>	MEAN PULMONARY VENOUS BLOOD PRESSURE <sup>2</sup>	PERIPHERAL VENOUS PRESSURE	CARDIAC INDEX
mm. Hg	mm. Hg $\pm \sigma$	mm. Hg $\pm \sigma$	cm. citrate $\pm \sigma$	l./m <sup>2</sup> /min. $\pm \sigma$
Below 100.....	(7)	(7)	(6)	(4)
Average—82.3.....	14.0 $\pm$ 3.4 (a)	2.3 $\pm$ 2.5 (a)(b)	2.7 $\pm$ 1.7	1.6 $\pm$ 0.5 (a)(b)
100–149.....	(29)	(30)	(29)	(16)
Average—130.0.....	16.7 $\pm$ 3.4	7.6 $\pm$ 3.9 (a)	3.0 $\pm$ 2.8	3.3 $\pm$ 1.1 (a)
Above 150.....	(17)	(19)	(20)	(6)
Average—164.8.....	18.5 $\pm$ 3.6 (a)	9.7 $\pm$ 4.5 (b)	3.7 $\pm$ 2.8	4.4 $\pm$ 1.5 (b)
Overall mean.....	(50)	(52)	(49)	(22)
100 and above.....	17.8 $\pm$ 3.6	8.5 $\pm$ 4.2	3.3 $\pm$ 2.4	3.6 $\pm$ 1.3
Average—144.2				

<sup>1</sup> Average values, number of cases in parenthesis.

<sup>2</sup> Pressures relative to the intrathoracic pressure as a base.

(a), (b):  $P = 0.01$  or less for the differences in means between two values marked (a) or (b). In other instances  $P = > 0.01$ . See text.

correlations were found in these experiments between systemic arterial and systemic venous pressures. This difference between the lesser and greater circulations is noteworthy in relation to the dynamics of the circulation.

As regards the cardiac outputs of dogs under nembutal, the values obtained show a highly significant positive correlation between arterial blood pressure and cardiac index. The animals with low blood pressures were not subjected to more hemorrhage or trauma than the others. Random differences in sensitivity to the anesthetic agent may account in part for the differences in blood pressure among the several animals.

The data on 26 animals in which cardiac output determinations were made have been analyzed with respect to the correlation between pulmonary venous pressure and cardiac index. Thirteen dogs had mean pulmonary venous pressures below 5 mm. Hg, and an equal number were above this value. The average cardiac

index for the former group was  $2.62 \pm 1.3$  l/m<sup>2</sup>/min. and for the latter  $4.00 \pm 1.2$ . The probability value for the difference is less than 0.01. Thus a positive correlation exists between left heart filling pressure (pulmonary venous pressure) and the output of the heart in the absence of signs of failure in the intact dog.

No significant correlations were found between systemic venous pressure and cardiac index, nor was there any correlation between body weight or sex and pulmonary venous and artery pressures. It should be noted, in connection with the absence of correlation between systemic venous pressure and cardiac index, that one is dealing with inter-individual variations in the 'normal' anesthetized state and not intra-individual variations with changes in conditions.

#### DISCUSSION

The integrated mean pulmonary artery pressures found in these experiments on anesthetized dogs are less than those reported by most other observers in unanesthetized animals. Our observations are, however, in fairly close agreement with those of Hellems, Haynes, Dexter and Kinney (8, 9). Their figures are, it may be noted, uncorrected for intrathoracic pressure.

The correlations shown in table 1 between systemic blood pressure and lesser circulation pressures must be interpreted in the light of the simultaneous correlation between cardiac index and pulmonary venous pressure. In general, low systemic arterial pressures were associated with low cardiac outputs. The low pulmonary venous pressures associated with the low outputs indicate that an inadequate filling pressure was probably the cause of the low output. It should be emphasized that the observations reported were made while the animals were in good clinical condition and before they had been exposed to any experimental procedures other than those involved in placing catheters and cannulae. When obvious left heart failure supervenes, either terminally or because of some experimental procedure, the pulmonary venous pressure rises without concomitant rise in cardiac output (15).

Thus it seems proper to conclude from the data reported that in the absence of signs of left heart failure the cardiac output is positively correlated with pulmonary venous pressure. The fact that no statistically significant correlation appeared between systemic venous pressure and cardiac output is, at least superficially, out of line with prediction from the Starling Law of the Heart. However, it is important to note that the systemic venous pressure was measured in the jugular vein at a greater distance from the heart, and further, that some degree of right heart failure may have existed in some instances without simultaneous left sided failure. Furthermore, the mean systemic venous pressure values for large cardiac outputs were actually higher than those for small outputs (table 1). The failure to show a statistically significant relation is, we believe, an indication that uncontrolled variables such as those mentioned above, or others, have complicated the situation. Furthermore, it must be emphasized again that we are dealing entirely with inter-individual variations in these data, and that in terminal heart failure (15) we have always observed the systemic venous pressure to rise, with no change or a fall in cardiac output.

Nevertheless, the fact that these data show a highly significant correlation between left heart filling pressure and cardiac output, without demonstrating the

same regularity for the right side of the heart, is of considerable interest. The result indicates that one is not justified in assuming that right and left venous filling pressures must vary proportionately. Further, it suggests that the left atrial and pulmonary venous pressures are apt to be raised more in association with high cardiac outputs in the absence of failure than is the systemic venous pressure. The greater mass and consequent greater resistance to filling of the left ventricle may be a factor in causing this difference.

#### CONCLUSIONS

Methods are described for measurement of pulmonary arterial and venous pressures in the anesthetized dog by a flexible catheter technique, simultaneously with cardiac output, intrathoracic pressure, systemic arterial and venous pressures. Left heart catheterization was attended by a mortality of about 10 per cent. Observations on 64 dogs are presented, showing significant positive correlations between systemic arterial pressure and *a*) integrated mean pulmonary artery pressure, *b*) integrated mean pulmonary venous pressure and *c*) cardiac index. A significant positive correlation was also found between mean pulmonary venous pressure and the cardiac index. Although the mean systemic venous pressure was higher in dogs with high than with low cardiac indices, the difference was not statistically significant.

These results have been discussed in relation to other known facts of cardiovascular physiology.

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# CIRCULATORY CHANGES AND PULMONARY LESIONS IN DOGS FOLLOWING INCREASED INTRACRANIAL PRESSURE, AND THE EFFECT OF ATROPINE UPON SUCH CHANGES<sup>1</sup>

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**P**ULMONARY edema and congestion occur frequently in cases of head injury (1-3). Weisman (4) found lung edema and congestion in approximately two-thirds of 686 cases of traumatic and spontaneous intracranial hemorrhage dying within 30 minutes to one hour after the injury. Conflicting reports (5-8) have appeared with reference to lung edema following bilateral cervical vagotomy. Recently Sussman *et al.* (9) maintained guinea pigs on artificial respiration and studied the importance of insufflation pressure in the genesis of pulmonary edema. They stated "under the conditions of these experiments there is no evidence that vagotomy exerts an influence on the pulmonary vascular system favoring edema or hemorrhage."

On the other hand Luisada and Sarnoff (10) produced pulmonary edema in dogs by the rapid intracarotid infusion of saline solution. These investigators (11) employed massive, rapid venous infusion in dogs simultaneously with vagal stimulation and concluded "electrical stimulation of either the cardiac end of the cu-vagi or the intact nerves favors pulmonary edema by causing extreme bradycardia."

Luisada and Sarnoff (12) also reported that parasympatholytic drugs promoted lung edema caused by the rapid intracarotid infusion of physiological salt solution, and they stated that atropine aggravated edema of the lungs under such conditions.

Recently 2 of the present authors (13) reported that in the guinea pig under artificial respiration bilateral cervical vagotomy provided great protection against the pulmonary edema produced by elevation of intracranial pressure. Surtshin, Katz and Rodbard (14) have questioned whether in previous attempts to produce lung edema by increased intracranial pressure the effects were due to genuine edema or were artifacts resulting from the aspiration of saliva. The possibility that this factor influenced the results in the experiments to be reported has been ruled out by the routine use of an endotracheal tube.

## METHODS

Mongrel dogs were anesthetized with intravenous pentobarbital sodium, 30 mg/kg., and a trephine opening one centimeter in diameter was made just lateral to the mid-calvarium in 18 dogs.

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The intracranial pressure of these animals was elevated by inflating with fluid a small latex balloon placed extradurally through the trephine opening. The volume of fluid introduced was adjusted to produce bradycardia and elevation of pulmonary venous pressure without apnea. A metal dome 1.5 centimeters in diameter, with an opening in its top just large enough to accommodate the plastic tube leading to the balloon, was securely fastened to the bony calvarium with two small screws. This dome held the inflated balloon in place.

Number 10 radio-opaque, whistle-tipped ureteral catheters were employed in right and left heart catheterization, and pressures were recorded in the pulmonary artery and a pulmonary vein respectively with standard resistance wire pressure transmitters (Statham strain gauges). The direct Fick principle was utilized in determining cardiac outputs, and the latter were expressed as cardiac indices ( $l/min/m^2$ ). A more detailed description of the technic employed may be found in the preceding paper (15).

Femoral arterial pressure was measured with a mercury manometer, and femoral venous pressure was measured with a manometer filled with 5 per cent sodium citrate. Heart rates were counted from the pulmonary artery pressure records. Integrated mean pulmonary vascular and intrathoracic pressures were measured with a compensating polar planimeter, and all pulmonary pressures reported in this paper are expressed in relation to intrathoracic pressure as zero.

All dogs had an indwelling tracheal cannula, and an intermittent positive pressure-blast respirator was used in certain experiments as designated in table 1. Atropine sulfate was injected intravenously in 4 dogs after increasing intracranial pressure.

## RESULTS

Figure 1 illustrates the results of a typical experiment in which increased intracranial pressure was induced. The systemic blood pressure fluctuated irregularly, but associated with the decline in heart rate and cardiac output there were marked elevations in pulmonary artery and vein pressures. The animal died shortly after the last readings and the lungs showed moderate congestion and edema of the dependent lobes and slight edema of the other lobes. The mean values for the variables in question in 18 such experiments are shown in figure 2. It will be noted that the rise in pulmonary venous pressure after one hour of increased intracranial pressure is about 10 mm. Hg, while the systemic venous pressure rise is approximately half as great. The pulmonary artery pressure rise is on the average 8 mm. Hg. The decline in cardiac output is about 30 per cent. The changes are progressive and maintained.

These results are to be contrasted with those obtained when atropine is administered 15 minutes after elevating the intracranial pressure. Figure 3 shows an example of such an experiment. It will be noted that the rise in pulmonary venous pressures was reversed and that the heart rate and cardiac output returned to normal or higher in spite of maintained, elevated intracranial pressure. The mean values obtained in 4 such experiments are shown in figure 4.

The pulmonary lesions found at necropsy are indicated in table 1, which also presents the data concerning mean pulmonary venous pressure after increasing the intracranial pressure. It will be noted that in general the lung edema was minimal in instances in which the pressure was low and greater when the venous pressure was high.

## DISCUSSION

This investigation yielded certain interesting phenomena common to all animals subjected to increased intracranial pressure. A significant elevation of mean

pulmonary venous pressure which occurred simultaneously with bradycardia, lowered cardiac output and slight to marked increase in mean pulmonary artery pressure

TABLE 1

WEIGHT	MEAN PULMONARY VENOUS PRESSURE		SURVIVAL TIME AFTER I.I.C.P.	ARTIFICIAL RESPIRATION	INDEX OF LUNG PATHOLOGY <sup>1</sup>
		Time after I.I.C.P.			
kg.	mm. Hg	hr./min.	hr./min.		
19.3 ♂	5.5	0:50	0:53 d <sup>1</sup>	—	0-1
9.3 ♀	6.0	1:05	2:45 d	—	0-1
27.0 ♀	11.5	3:00	3:46 d	—	0-1
12.1 ♀	12.0	1:10	1:50 d	—	0-1
13.2 ♀	12.0	1:13	2:30 d	—	2
23.6 ♂	12.8	1:33	2:35 d	—	2
12.7 ♂	18.5	0:05	0:15 d	—	0-1
25.8 ♂	18.5	0:10	0:35 d	—	2
25.8 ♂	19.0	1:07	1:25 d	—	2
12.0 ♂	21.0	1:48	1:58 d	+	2
17.3 ♂	23.3	1:47	1:54 d	—	2
22.9 ♂	25.0	2:40	2:42 d	—	2
16.1 ♀	26.0	0:48	0:55 d	—	2
26.3 ♂	31.0	0:55	1:03 d	—	2
22.7 ♂	11.5 <sup>1</sup>	0:05	0:50 d	+	0-1
	5.3 <sup>2</sup>	0:08			
25.9 ♀	13.3 <sup>1</sup>	0:07	1:04 s <sup>1</sup>	+	0-1
	3.8 <sup>2</sup>	0:11			
28.2 ♂	13.8 <sup>1</sup>	0:13	0:58 s	+	0-1
	8.5 <sup>2</sup>	0:18			
25.0 ♂	17.0 <sup>1</sup>	0:03	1:45 s	+	0-1
	9.0 <sup>2</sup>	0:04			

<sup>1</sup> Increased intracranial pressure.

<sup>2</sup> 1) Slight edema, congestion, and hemorrhage involving only the dependent portion of the lungs.

2) Moderate edema, congestion and hemorrhage involving only the dependent portion of the lungs with slight involvement in other portions.

3) Marked edema, congestion, and hemorrhage involving only the dependent portion of the lungs with moderate involvement in other portions.

4) Frank edema, congestion and hemorrhage involving all the lobes of the lungs.

<sup>3</sup> Died. <sup>4</sup> Prior to atropine administration. <sup>5</sup> After atropine administration. <sup>6</sup> Killed.

was a uniform finding. From these data it is apparent that the hydrostatic pressure rose within the pulmonary capillaries. Although the fluctuations in systemic arterial pressure were rather small, there was a marked increase in pulse pressure associated with the bradycardia.

The results of these experiments show that the magnitude of the increase in pulmonary venous pressure is correlated with the extent of pulmonary edema.

They do not, however, prove a causal relationship. Two relevant facts must be noted. First, it is seen that pulmonary edema may occur at venous pressure levels lower than the usual colloid osmotic pressure of dog plasma. However, since from

Fig. 1. CHANGES IN CARDIAC INDEX, heart rate and pressures in pulmonary artery, pulmonary vein and femoral artery, in relation to elevation in intracranial pressure in a nembutal anesthetized dog.

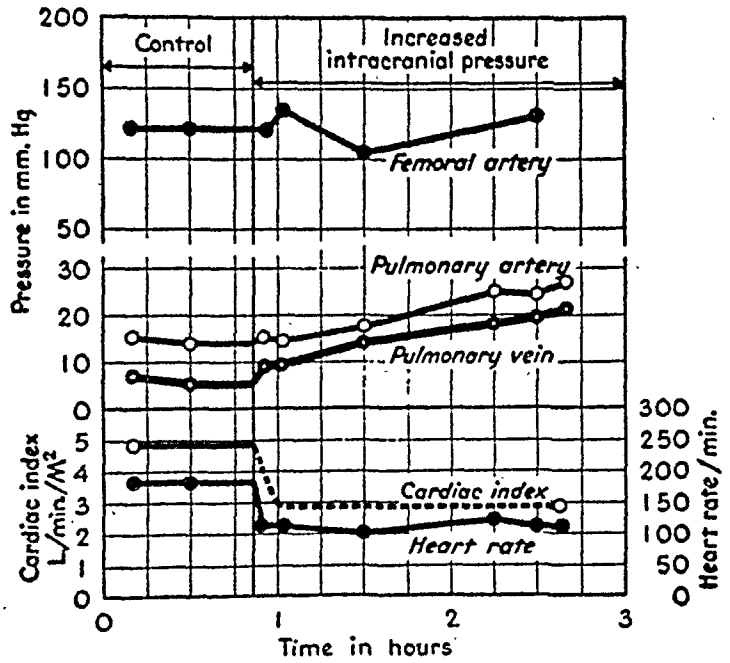
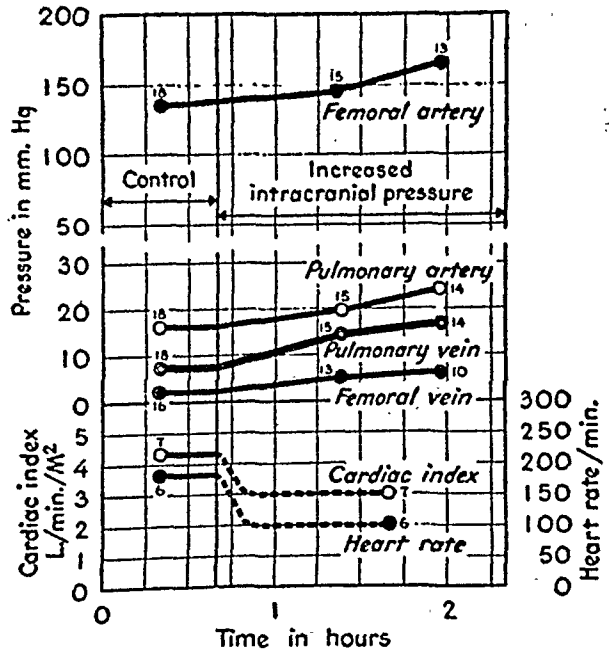


Fig. 2. MEAN VALUES FOR C.I., H.R. and pressures in P.A., P.V., F.A. and F.V. in relation to elevation in I.C.P. in nembutalized dogs. The figures at each point represent the number of dogs included in each mean value at a particular time.



1000 to 2500 cc. of 5 per cent dextrose solution was administered via the catheters by drip to prevent blood clotting in them, there may have been a lowering in plasma protein concentration. This was not measured. Second, the higher venous pressures did not produce maximal edema in the time of study in any case. In the case of the highest pulmonary venous pressures recorded they were within 4 to 10 minutes of the termination of the experiment and insufficient time may have been allowed to

give maximal edema. The results of these experiments taken as a whole indicate that an elevation in effective filtration pressure in the lung capillaries is probably the major factor in the genesis of pulmonary edema after increased intracranial

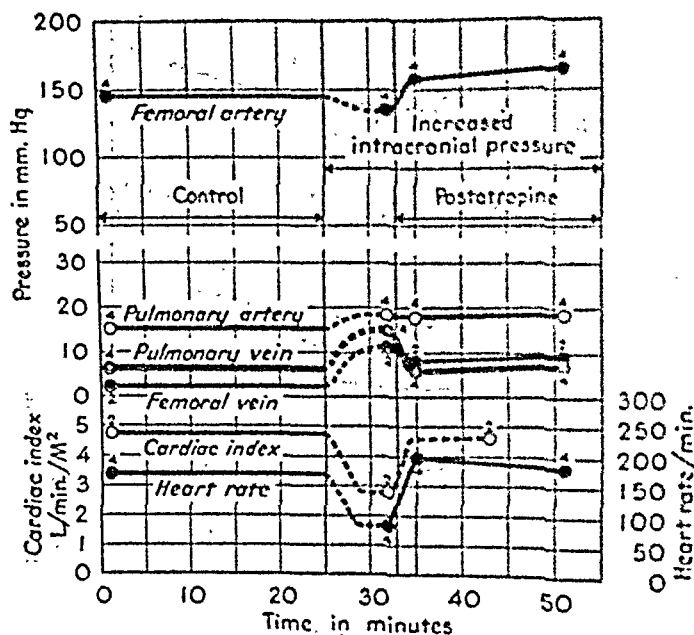
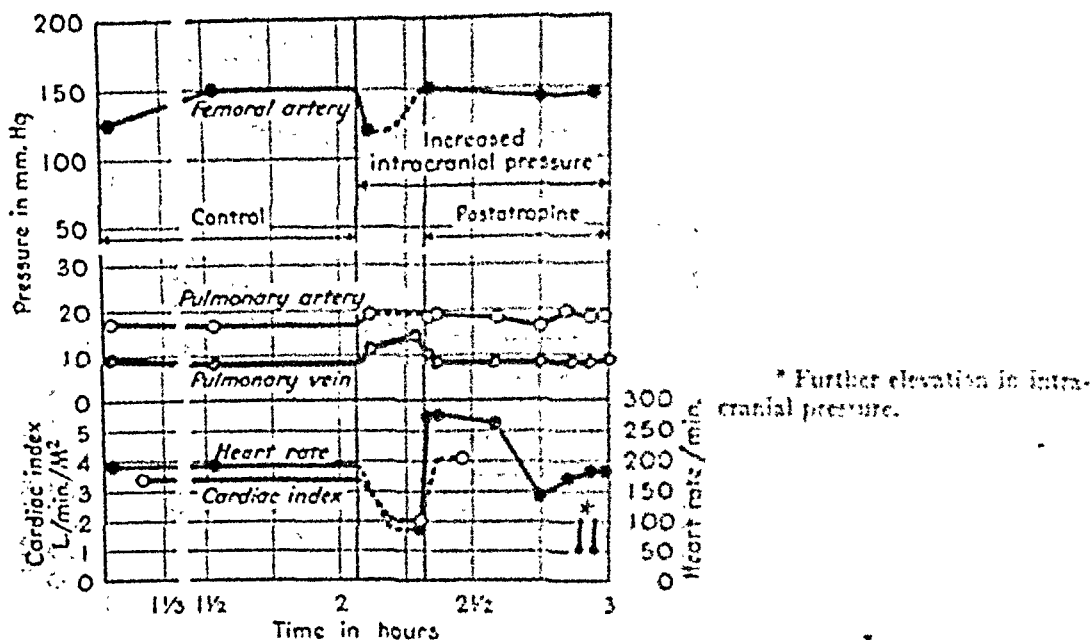


Fig. 4. MEAN VALUES FOR C.I., H.R. and pressures in P.A., P.V., F.A. and F.V. in relation to increased intracranial pressure in nembutalized dogs, plus the effect of atropine upon these changes. The figures at each point represent the number of dogs included in each mean value at a particular time.

pressure. The fact that atropine increased the heart rate and cardiac output, and lowered the pulmonary venous pressure, and that furthermore, after these changes the lungs were practically free of edema, is important. It is perhaps the most impressive point in favor of the view that the pulmonary edema otherwise seen after increased intracranial pressure may be the direct consequence of the elevated venous, and by inference the capillary, pressures.

only the fasting invertase output of *dog 1* and the fasting invertase and peptidase output of *dog 2* are significantly higher than the controls. The intravenous saline increased the volume of the intestinal juice over that of the other control experiments. The increase in volume was especially great in *dog 1* and the changes are significant. The enzyme production was augmented but the change was not statistically significant.

#### DISCUSSION

Generally it can be stated that parathyroid extract, vitamin D, and calcium gluconate all diminish the volume of intestinal juice. The parathyroid extract and vitamin D also depress the enzyme output thus setting them apart from calcium gluconate which tends to augment enzyme production.

The diminished volume output of intestinal juice during hypercalcemia parallels closely the results obtained with parathyroid extract and vitamin D by Babkin *et al.* (1) and Schiffrin (2) in dogs with innervated, but not denervated, gastric pouches. This correlation suggests that the diminution of secretion is mediated by way of the extrinsic nerves. Grant (11), however, reported that injections of calcium lactate or calcium chloride in sacrifice experiments inhibited both the nervous and chemical phases of gastric secretion.

It is well known that an increase in calcium concentration will decrease cellular permeability especially to water (12, 13). Asher and Jost (14) and Engel (15) have shown that sympathectomy decreases capillary permeability, and according to Bronk *et al.* (16) and Harvey and MacIntosh (17) high calcium concentration blocks transmission of nerve impulses in ganglionic synapses. Thus, calcium can bring about a decrease in cellular permeability both directly and indirectly through the sympathetic nervous system. According to Feng (18) transmission across the neuromuscular junction is inhibited by excess calcium ions. It has been shown by Solandt (19) that treatment of a nerve with a solution containing a high concentration of calcium lowers the time-constant of accommodation and, therefore, would tend to decrease the number of impulses reaching the intestine by the extrinsic nerves.

Schiffrin (2) reported that during hypercalcemia, pepsin output was increased in innervated gastric pouches but that no change occurred in the denervated preparations. From the results obtained with innervated intestinal segments, it is evident that only the hypercalcemia due to calcium gluconate injection caused any rise in enzyme production, and this was confined to the fasting animal.

Calcium ions seem to accelerate the activity of several enzymes (20, 21). Delzenne (22) stated that dilute solutions of calcium chloride increase and concentrated solutions diminish the proteolytic activity of pancreatic juice. This could hardly explain the difference in the enzyme activity of the intestinal juice during hypercalcemia because the calcium content of the intestinal juice is subject to considerable normal variation (1.0 mM/l. to 6.2 mM/l.) without any correlation with enzyme activity. The difference in enzyme output in experiments with parathyroid extract and vitamin D on the one hand and calcium gluconate on the other may be due to the rate of development of the hypercalcemia. In the calcium glu-

conate experiments the rise in blood calcium was very rapid, whereas in both parathyroid and vitamin D experiments, which required mobilization of calcium from endogenous sources, hypercalcemia developed rather slowly.

Vitamin D is reported to assist in the absorption of calcium from the intestine (23-25) and to increase the acidity of the intestinal contents (26, 27). The results reported here for the pure intestinal juice show that there is no significant change in the pH chloride, or carbon dioxide capacity of the intestinal juice during vitamin D administration.\*

#### SUMMARY

Hypercalcemia was produced in the dog by the administration of parathyroid extract, calciferol, and calcium gluconate and observations made on the secretion of intestinal juice by loops of jejunum with intact extrinsic innervation. The administration of parathyroid extract subcutaneously, or calciferol by mouth, resulted in diminished volume of juice and total output of enzymes. The injection of calcium gluconate intravenously resulted in diminished volume of juice but an increased total output of enzymes. It is suggested that the change in volume of juice may be due to diminished excitability of the extrinsic nerves and a decrease in permeability of the gland cell membranes.

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# ROLE OF THE CENTRAL NERVOUS SYSTEM IN THE BODY TEMPERATURE-ARTERIAL PRESSURE RELATIONSHIP<sup>1</sup>

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PREVIOUS studies have called attention to a relationship between the body temperature and the arterial blood pressure which may be seen in the turtle (1), the chicken (2) and the dog (3). This relationship is seen in a fall in blood pressure which occurs during profound lowering of the body temperature, and in the return of the pressure to control levels when the animal is rewarmed. Other evidence suggests that the temperature-pressure (thermobaric) relationship holds generally among vertebrates including representative amphibians (frog, 4), reptiles (turtle, 5), mammals (3) and birds (chicken, 2, and pigeon 6).

Certain general limitations have been found to restrict the relationship. For example, when an animal is warmed above a critical temperature level dependent upon the species a lethal fall in blood pressure ensues. These critical levels appear to be about 3°C. above the normal value in warm blooded animals. In the turtle the upper limit varies from 30° to 37°C. and in the frog it is about 25°C.

In the present study several phases of the thermobaric relationship were investigated. Data were obtained concerning the pressure levels after cooling or warming when the animal is maintained at a constant thermal level for several hours. The mechanism of the thermobaric relationship was also investigated. Conceivably this mechanism may operate via changes in the heart rate, in the reactivity of the blood vessels, or via the intermediation of the central nervous system through the parasympathetic or sympathetic outflow. The responsiveness of the vessels at various body temperatures was assayed by testing the vasoconstrictor effect of an intravenous injection of epinephrine. The role of the central nervous system was assayed by observing the blood pressure response to warming and cooling before and after sectioning of the vagi, destruction of the brain, or section of the spinal cord. Heart rate and blood pressure responses were compared under these various circumstances.

## METHODS

The large blood vessels were exposed in 68 turtles (*Pseudemys elegans*) by drilling a 2½-cm. hole with a circular saw in the plastron at the level of the pectoral plate. A glass cannula was inserted caudad into the left aorta to measure the peripheral systemic arterial pressure. Such a procedure is possible in the turtle because of the bilateral aortae, which join below the transverse septum. A thermometer was inserted and tied into the cloaca for the measurement of the body temperature.

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<sup>3</sup> The department is supported in part by the Michael Reese Research Foundation.

The animal was taped to a small board and placed in a water bath at room temperature. The cannula was then connected to the Hamilton manometer (7) for optical recording of the blood pressure and heart rate.

One group of animals was maintained at a constant temperature level ranging between 5° and 40°C. for periods up to 3 hours. In a second group the effect of bilateral vagotomy on the thermobaric relationship was determined. In a third group, consisting of 18 animals, the spinal cord was cut above the level of the first cervical vertebra (C<sub>1</sub>) by flexing the head and inserting a knife immediately inferior to the occipital suture. The gross lesions were checked at the end of the experiment after a period of time up to an hour in 15 of these animals and of 24 hours in 3; the turtles were then cooled and rewarmed to determine the blood pressure response to temperature under these conditions of interruption or destruction of the brain stem.

The response to epinephrine was determined before and after the section of the vagi or the spinal cord since this presented a technique for the assay of the responsiveness of the peripheral vessels to vasoconstrictor materials under the conditions of our experiment.

*Time Factor in the Temperature-Pressure Relationship.* Although a general relationship between body temperature and blood pressure is usually demonstrable during cooling or warming no data have been available concerning the persistence of the pressure changes when the animal is maintained for an extended period at a given temperature. Five groups of 3 turtles each were warmed or cooled from room temperature to levels at 5°, 15°, 25°, 30° and 35°C. and maintained at these levels for periods up to 3 hours. Blood pressures changed to some extent during the change in body temperature. However, no consistent changes in blood pressure were seen during the extended period at the constant temperature. Two other groups of animals maintained at 38° or 40°C., respectively, showed a progressive fall in blood pressure as the experiment continued. Representative average data on 4 of these groups are given in table 1.

At the end of each of these experiments, each animal was injected with 1 mg. of epinephrine in 1 cc. of saline and the blood pressure changes were recorded. An epinephrine response was obtained in all groups suggesting that the blood vessels were still capable of responding to a stimulus despite the fact that in the animals at 38° and 40°C., the blood pressure had already fallen to low levels (table 1). In the group at 5° the response was slow and long-persisting as compared with the groups at higher temperatures. This is in accord with other reports that drug action is retarded at lower temperatures (8).

*Effect of Vagotomy.* In order to determine the possible role of the parasympathetic system as represented by the vagi, the effect of these nerves on the thermobaric relationship was studied in a series of 8 turtles. Vagotomy was usually followed immediately by a transient slight increase in heart rate from an average of 38 to 50. After a few minutes the heart rate returned to essentially control values. Vagotomy had no significant effect on the blood pressure over a period of 160 minutes during which the animals remained at room temperature, about 22°C.

Cooling, in a series of 7 turtles, resulted in a moderate fall in blood pressure, and rewarming was followed by an equal rise. The heart rate also changed with the temperature, falling during the cooling period and rising during the rewarming. The average values for these two groups of experiments are given in table 2. These results indicate that the vagi probably played little role in the body temperature-blood pressure mechanism.

*Effect of Section of the Spinal Cord.* The possible role of the central nervous system as represented by the spinal cord outflow in the thermobaric relationship could be tested by section of the spinal cord high in the neck. Since this procedure is known to affect the blood pressure, it was necessary to quantitate this effect in order to have a baseline for further work. For this purpose the cord was cut at about the first cervical segment in 14 turtles, and these were maintained at a constant temperature for a period of 60 minutes after section of the cord. Spinal reflexes returned

TABLE 1. EFFECT OF MAINTAINED BODY TEMPERATURE ON BLOOD PRESSURE

BODY TEMPERATURE	5°C.			15°C.			30°C.			40°C.		
	Blood pressure		Heart rate/min.	Blood pressure		Heart rate/min.	Blood pressure		Heart rate/min.	Blood pressure		Heart rate/min.
	Sys-tolic	Dias-tolic		Sys-tolic	Dias-tolic		Sys-tolic	Dias-tolic		Sys-tolic	Dias-tolic	
	mm. Hg	mm. Hg		mm. Hg	mm. Hg		mm. Hg	mm. Hg		mm. Hg	mm. Hg	
Immediate.....	19	12	8	37	31	19	46	34	29	28	13	38
1 hour.....	17	12	7	35	28	14	39	32	36	19	11	33
2 hours.....	18	13	6	30	28	24	44	33	40	15	5	21
3 hours.....				29	25	24	38	29	31			
Change due to epinephrine.....	+15	+11	+8	+42	+31	-10	+35	+26	-6	+13	+16	+33

TABLE 2. EFFECT OF BILATERAL VAGOTOMY IN TURTLES

	KEPT AT CONSTANT TEMPERATURE (22°C.)			WITH TEMPERATURE VARIATIONS			
	Blood pressure		Heart rate/min.	Tempera- ture	Blood pressure		Heart rate/min.
	Systolic	Diastolic			Systolic	Diastolic	
	mm. Hg	mm. Hg		mm. Hg	mm. Hg		
Control.....	32	22	38	22°C.	27	21	46
	<i>Bilateral vagotomy</i>				<i>Bilateral vagotomy</i>		
5 min.....	31	25	50	10°C.	22	19	27
20 min.....	30	23	34	20°C.	28	23	32
40 min.....	29	23	41	30°C.	31	27	60
60 min.....	28	23	38				
160 min.....	28	23	43				

almost at once, showing, as is well known, the very short duration of spinal shock in poikilotherms.

The blood pressure began to fall within a minute after section of the cord and became relatively stable in 15 to 30 minutes at an average of 16/11 mm. Hg (tables 3 and 4). The fall in blood pressure was more gradual in the animals kept at 10°C. than in those at higher temperatures. Epinephrine (1 mg. in 1 cc. of saline) was then injected into the aorta causing a marked rise in pressure similar to that seen in the normal animal, showing that the blood vessels were still capable of responding to such pressor stimuli. The heart rate was generally unaffected by either the cord section or the injection of epinephrine.

*Effect of Temperature Change After Cord Section.* For the purpose of the present experiments the spinal cord was cut at the first cervical level in 12 animals and a period of 30 minutes allowed to elapse in order to permit the pressure to stabilize at the new lower values seen consistently after section of the cord (table 3). The animals were then cooled and rewarmed as before and blood pressures and heart rates were recorded at intervals. It was seen that although the heart rate usually increased somewhat at higher temperatures and decreased at lower temperatures, the blood pressure did not change significantly. Average blood pressures and heart rates with

TABLE 3. EFFECT OF CORD SECTION ON BLOOD PRESSURE<sup>1</sup>

BODY TEMPERATURE	15°C.		25°C.		35°C.	
	Systolic	Diastolic	Systolic	Diastolic	Systolic	Diastolic
	mm. Hg	mm. Hg	mm. Hg	mm. Hg	mm. Hg	mm. Hg
Control.....	31	24	31	22	36	27
5 min. after cutting cord.....	32	27	22	16	18	13
15 min. after cutting cord.....	29	24	14	9	13	8
30 min. after cutting cord.....	20	13	11	7	13	8
60 min. after cutting cord.....	15	10	8	4	12	9
Maximum press. after epinephrine.....	36	30	44	37	39	32

<sup>1</sup> Averages taken on 5 turtles at each temperature.

cooling and warming on 12 turtles are given in table 3. Similar results were obtained in 3 turtles in which the spinal cord was sectioned 24 hours before the thermal testing, and in 8 other turtles after pithing of the brain.

#### DISCUSSION

These results indicate that the thermobaric relationship is maintained when the animal is kept at constant temperature for periods up to 3 hours. Thus earlier findings that the blood pressure changes with body temperature in the poikilotherm (4, 9) are substantiated and shown to be dependent upon the temperature per se, rather than upon the rate or direction of temperature change. On cooling from room temperature (about 22°C.) to about 10°C., the blood pressure does not always follow the temperature change, but further cooling will bring out the depressor effect. The significance of this is unknown. However, warming within physiological limits almost always results in an increase in the blood pressure. These higher blood pressures seen at higher body temperatures may play a role in increasing the rate of delivery of blood to the tissues when metabolic activity is increased. At higher thermal levels, 38° and 40°C., the preparation deteriorates rapidly, as shown by the fall in blood pressure. The response to epinephrine persists at these temperatures, although reduced in intensity. The constrictive properties of the blood vessels are therefore not markedly impaired. These results suggest that coordinating mechanisms which adjust blood pressure to body temperature are probably not able to function adequately at such high temperatures. Some aspects of lethal temperature levels are discussed in another communication (10).

Sections of the vagi which might affect both heart rate and splanchnic vasomotion do not significantly affect the thermobaric relationship. Section of the spinal cord or pithing of the brain eliminated the effect of the body temperature change on the blood pressure. This occurs despite the fact that the heart rate may continue to increase with increasing body temperatures. The response to epinephrine injection is also unchanged after section of the cord. It would therefore appear that in the turtle, the temperature-pressure relationship is independent of changes in the heart rate or the reactivity of the blood vessels to exogenous vasoconstrictive agents. Instead, the relationship apparently depends upon the integrity of the central nervous system.

These data on cord section and on pithing may thus be considered as evidence for the presence in poikilotherms of a thermosensitive mechanism which produces adjustments of the blood pressure in response to thermal stimulation. Such a mechanism

TABLE 4. EFFECT OF BODY TEMPERATURE CHANGE ON BLOOD PRESSURE OF TURTLES WITH SECTION OF CERVICAL SPINAL CORD

BODY TEMPERATURE	CONTROL			AFTER CORD SECTION		
	Blood pressure		Heart rate/ min.	Blood pressure		Heart rate/ min.
	Systolic	Diastolic		Systolic	Diastolic	
	mm. Hg	mm. Hg		mm. Hg	mm. Hg	
10°C.	20	13	12	15	10	16
20°C.	28	20	30	17	11	22
30°C.	32	23	54	16	11	27
35°C.	38	32	80	17	12	44

may operate via either afferent impulses from the periphery or possibly by a direct thermal stimulation of the brain. In poikilotherms, such a mechanism obviously does not regulate the body temperature. Instead it may have other functions producing changes in the internal economy which permit more adequate adjustments to varying thermal levels to which such animals are repeatedly exposed.

The fall in arterial pressure to low levels seen on destruction of the brain or on sectioning of the cervical spinal cord is evidence for the tonic control of the blood pressure by the central nervous system. The present studies suggest that such tonic control of the blood vessels may be varied in response to thermal stimuli, either via temperature receptors in the periphery or perhaps by a direct effect on a central thermoceptive mechanism.

#### SUMMARY

An increase or decrease in the body temperature of the turtle results in covariance of the systemic arterial blood pressure. These changes persist for periods of at least 3 hours, if the body temperature is kept constant. At higher temperatures (38° to 40°C.) the pressure is not maintained, but falls to low levels and death ensues. Section of the vagi has no effect on the relationship. Destruction of the brain or section of the cervical spinal cord at the occiput results in a fall in pressure to levels of about 16/11 mm. Hg. After this procedure, warming or cooling of the animal no

longer results in a change in blood pressure. These experiments are considered as evidence for the dependence of the temperature-pressure relationship upon a mechanism residing in the central nervous system.

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# DENERVATION AND THE EXCITABILITY OF CERTAIN MUSCULAR EFFECTORS

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DENERVATED structures are more sensitive to chemical agents; this phenomenon, known as the law of denervation, was pointed out by Cannon (1) in 1939. There are some exceptions to this law. Ergotoxine and histamine produce a greater contraction on the normal nictitating membrane than on the denervated (2). The response of the soleus muscle to KCl is increased by prostigmine only in the denervated muscle, which is no more sensitive to KCl than the normal (3). Altamirano, Fernandez and Luco (4) pointed out that the ergotamine and dehydroergotamine contraction of the nictitating membrane is larger on the normal than on the denervated side and that the depressor action of atropine and curarizing drugs is more pronounced on the normal side; the same thing happens on the superior cervical ganglion. It seemed of interest to us to determine whether there are other chemical agents that show exceptions to the denervation law.

## METHODS AND RESULTS

Cats weighing 2 to 3 kg. were used, anesthetized with 1 cc/kg. of body weight with a 25 per cent solution of urethane (Merck), which contained 3.3 per cent of sodium pentobarbital (Nembutal, Abbott). Both smooth (uterus) and skeletal muscle were used. The uterus was denervated 9 to 18 days previously (5). Kymographic records were made by Cannon and Rosenblueth's method (6). Drugs were given by vein.

Most of the experiments on skeletal muscle were made on the quadriceps denervated 53 to 126 hours and 6 to 22 days previously; the tibialis and the soleus muscles were also employed, having been denervated 6 to 14 days previously. The femur or the tibia was fixed by means of drills as necessary. The muscular contractions were recorded by fastening the corresponding tendon to the short end of a lever loaded with rubber bands. Drugs, in these cases, were injected rapidly in the abdominal aorta below the inferior mesenteric artery, the lower aorta being ligated. When the quadriceps muscle was used, the femoral artery just below the deep femoral artery was also ligated. The kymographic record of both structures was made with levers of the same length and with the same tension, although the tension of the normal side was sometimes a little greater than that on the denervated. The controls used were the uterine horn and the homologous contralateral muscles crushed centrally. The trachea was cannulated for artificial respiration when necessary.

Drugs employed were adrenaline hydrochloride, 1:1000; histamine hydrochloride,

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ride, 0.5:1000; barium chloride (Merck), 1:150; pituitrin (pituitrol, Sanitas), 10/cc.; ergotamine (gynergene, Sandoz), 1:1000; prostigmine (Roche), 1:2000; caffeine, in 20% solution; veratrine, 1:5000; and intocostin (Squibb), 200/cc. The veratrine and ergotamine were used in alcoholic solution, the other drugs being dissolved in dis-

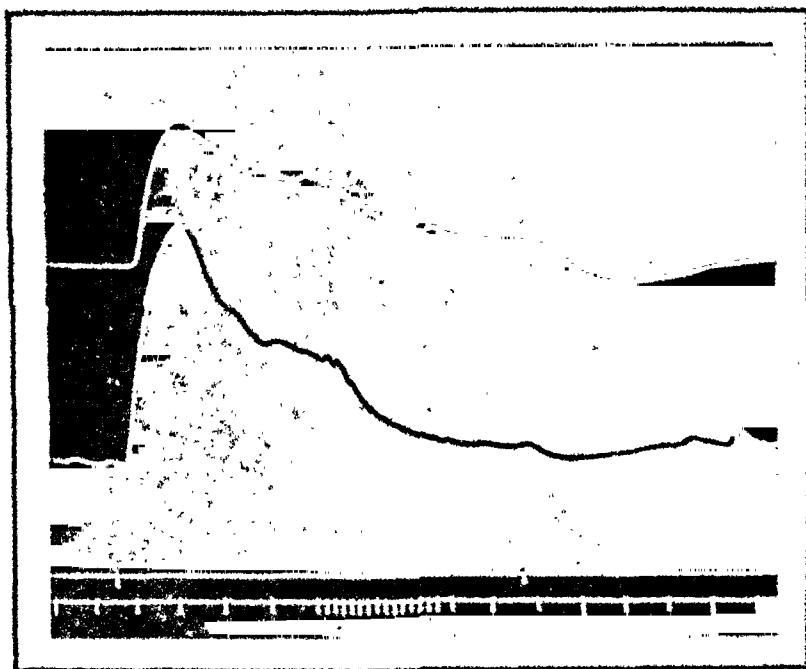


Fig. 1. GREATER EFFECT of pituitrin on normal uterine horn. *A*, Denervated (14 days) uterine horn; *B*, normal uterine horn. First signal: 0.5U pituitrin in femoral vein. Second signal: 2 micrograms of adrenaline i.v. In this as in other figures, time in minutes (variations in spacing of time signals are due to changes in speed of kymograph).



Fig. 2. GREATER EFFECTS of histamine and barium chloride on normal uterine horn. Upper record: uterine horn denervated 15 days before. Lower: normal horn. First signal, 1.5 micrograms of histamine; second, 2 mg. of barium chloride; both into the femoral vein.

tilled water. The volume injected was never more than 1 cc. and in general was 0.5 cc. more or less. If one animal was injected with more than one drug a sufficient time was permitted to elapse before the next drug was employed. Drugs were employed in threshold doses, or slightly in excess thereof, because the maximal effects may be the same in normal or denervated structures (1). If the doses were greater

than the threshold ones, their action was far greater than the structure was capable of developing.

**Smooth Muscle.** A base line joining the bottoms of the spontaneous contraction tracings of the uterine muscle was used to determine the height of the contraction produced by the drug. The initial level was restored for continuing the experiment if the tonus of the preparation changed during the procedure. Drugs employed were ergotamine, pituitrin, barium chloride and histamine.

Ergotamine in doses of 0.75 to 1 mg. always produced a greater effect on the normal uterine horn. The same thing happened when pituitrin was employed in doses of 0.4 to 1U (fig. 1). Barium chloride (1.6-3.6 mg.) produced greater contraction in

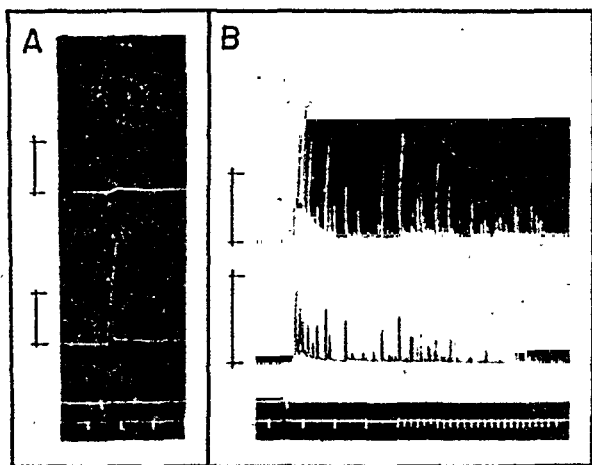


Fig. 3

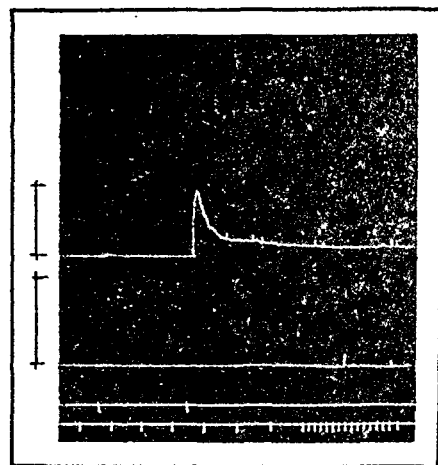


Fig. 4

Fig. 3. GREATER SENSITIVITY OF NORMAL MUSCLE to caffeine. A, Upper record: quadriceps denervated 6 days before; lower: normal muscle. At the signal, 0.15 gm. of caffeine intra-aortically. B, Upper record: normal quadriceps. Lower: quadriceps denervated 8 days before. At the signal: 0.16 gm. of caffeine. At left of each graph, 80-gm. tension.

Fig. 4. GREATER SENSITIVITY OF NORMAL MUSCLE to veratrine. Upper record: normal quadriceps. Lower: quadriceps denervated 8 days before. First and second signals: 0.5 and 1 mg. of veratrine. Left of each graph, 80 gm. tension.

the normal than on the denervated uterine horn (fig. 2). With histamine in doses of 1 to 3 micrograms we have obtained the same results in 7 experiments out of 9 (fig. 2).

Adrenaline was used to test the state of denervation. Luco's results (5) were confirmed. In 7 of 9 experiments performed a greater relaxation was observed on the denervated uterine horn in comparison with the control side.

**Skeletal Muscle.** 1) *Action of drugs on chronically denervated muscle in comparison with the homologous contralateral muscle crushed immediately before experiments.*

**CAFFEINE.** Caffeine injected rapidly into the terminal aorta in doses of 0.10 to 0.16 gm., in a volume of about 0.6 cc., produced a shortening in the normal as in the denervated muscle. In the kymographic record this shortening frequently took a tetanus-like form (fig. 3A), but sometimes irregular twitches appeared superimposed, as illustrated in figure 3B. The sensitivity to the drug of both muscles (normal and denervated after 14 days) was not the same. The threshold dose to produce any effect was smaller for the denervated structure (fig. 3). This was found in 7 of 8 experiments (table 1).

**VERATRINE.** The veratrine effect was studied on the quadriceps; 1 to 2 mg. was injected intra-aortically. In these conditions the drug produces a muscular shortening with a slow relaxation; during a prolonged time a certain degree of tension can be maintained (fig. 4). Like caffeine, veratrine sometimes produces irregular twitches superimposed on the tension induced by itself.

If the dose is small, it is possible to observe only a muscular shortening on the normal side. Greater doses are necessary to obtain a muscular response in the denervated

TABLE 1. ACTION OF CAFFEINE, VERATRINE AND PROSTIGMINE ON NORMAL AND DENERVATED EXPLANT MUSCLE

MUSCLE	DAYS DENERVATED	CAFFEINE		VERATRINE		PROSTIGMINE	
		N.	D.	N.	D.	N.	D.
Tibial	6					+	
Soleus	6					+	
Tibial	7					+	
Soleus	7					+	
Tibial	8						+
Tibial	9					+	
Soleus	11						+
Soleus	12	+					+
Tibial	12						+
Soleus	13					+	
Soleus	13		+				+
Tibial	13						+
Soleus	14	+					+
Quadriceps	6	+		+		+	
	6					+	
	7	+		+			
	8	+		+		+	
	8					+	
	9	+		+			+
	11	+		+		+	
	13			+			+
	15				+	+	
	15			+			+
	17			+			+
	20						+
	22			+			+

N, Normal side. D, Denervated side. +, Side of greater sensitivity.

vated (22 days previously) structure (fig. 4). This was the rule in 9 of 10 experiments (table 1).

**PROSTIGMINE.** The doses employed intra-aortically ranged between 25 and 500 micrograms. The mean volume injected was 0.20 to 0.40 cc. In the normal muscle a small dose of prostigmine produced fascicular twitches. A greater dose produced a tetanic contraction in which the fascicular twitches appeared superimposed. In the denervated muscle prostigmine caused only a tetanic contraction. The origin of fascicular twittings is not perfectly understood. However, some nervous mechanism is accepted because they vanished with denervation and because the prostigmine

produced discharges of the motor nerve (7). For these reasons the fascicular twitches have not been taken into account in the comparison of the sensitivity of normal and denervated muscles to the drug.

If the sensitivity of muscles is judged by the presence or by the height of tetanic contractions induced by prostigmine, it must be appreciated that the sensitivity depends on time of denervation and the muscle studied. Normal tibial and soleus muscle is more sensitive to the substance until the eighth or ninth day of denervation (fig. 5A); if the denervation time is longer, the denervated muscle is more sensitive (table 1). A similar course was observed with quadriceps muscle; for 12 days (mean

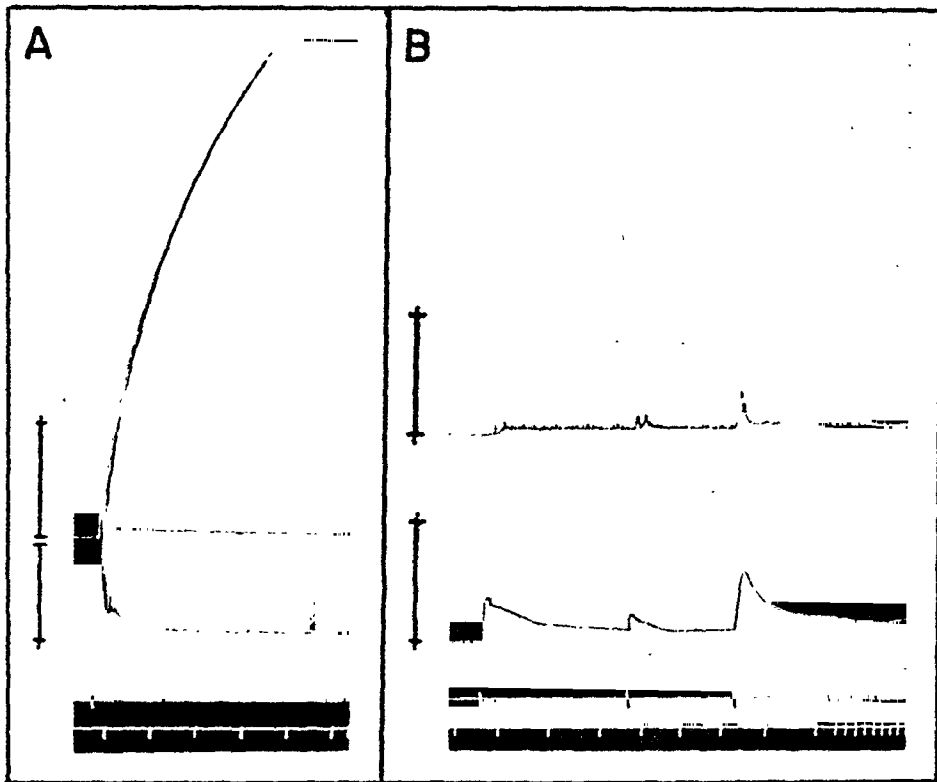


Fig. 5. GREATER SENSITIVITY OF NORMAL MUSCLE to prostigmine and inversion of this effect by a prolonged time of denervation. A, Upper record: tibial muscle denervated 6 days before; lower record: normal muscle. At the signal: 300 micrograms of prostigmine. B, Upper record: normal quadriceps. Lower: quadriceps denervated 15 days before. At the signal: 70, 50 and 250 micrograms of prostigmine. At left of each graph, 80-gm. tension.

time) the normal muscle is more sensitive, and after this time of denervation, the denervated one becomes the more sensitive (fig. 5B and table 1).

2) *Action of drugs on the chronically denervated muscle in comparison with the homologous contralateral structure crushed for 126 hours.* As pointed out above, the sensitivity of normal and denervated muscles to prostigmine depends on time of denervation; generally, the normal muscle is more sensitive, but when the time of denervation is longer, the denervated muscle becomes more sensitive. The explanation of this phenomenon is as follows: the result of the nerve stimulation by prostigmine (7), in addition to its action on the muscle (8), has a constant value; on the contrary, the prostigmine effect on the denervated muscle increases in relation to the denervation time. When the first two factors are greater than the second (sensitization by de-

TABLE 2. COMPARISON OF SENSITIVITY OF RECENTLY DENERVATED QUADRICEPS MUSCLE WITH THE HOMOLOGOUS CONTRALATERAL CHRONICALLY DENERVATED STRUCTURE

TIME DENERVATED		PROSTIGMINE		VERATRINE		CAFFEINE	
R.	C.	R.	C.	R.	C.	R.	C.
hr.	days						
53	6	+					
76	7		+				+
77	7		+	+			
77	7		+				
101	8		+				
125	10		+		+		+
78	11		+		+		+
102	12				+		+
126	13		+		+		+

R, Recently denervated muscle.  
greater sensitivity to the indicated drugs.

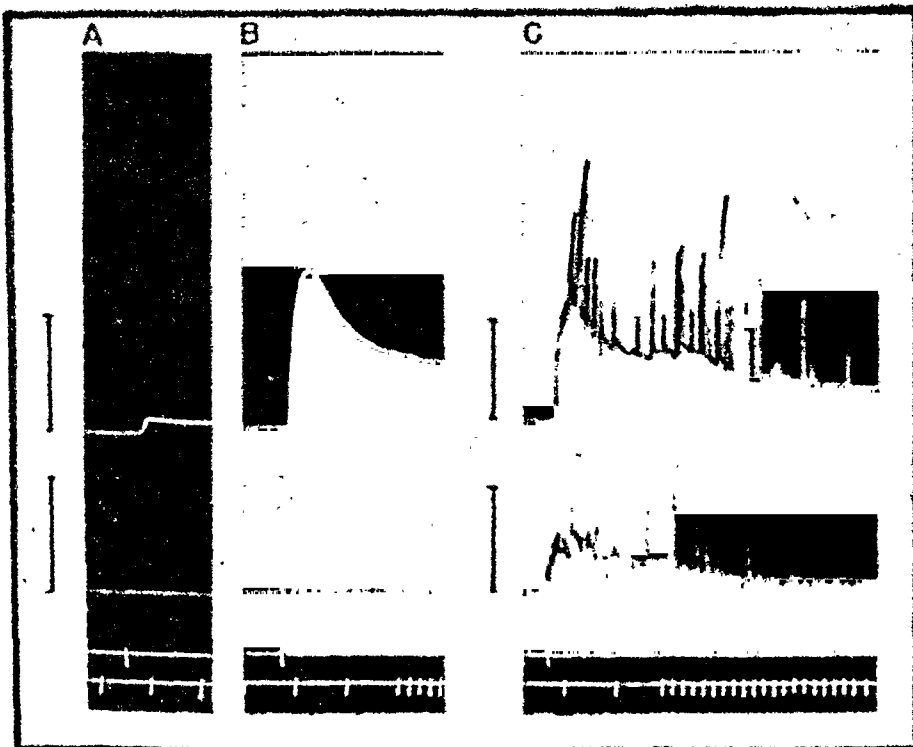


Fig. 6. GREATER EFFECT OF CAFFEINE, prostigmine and veratrine in the chronically denervated muscle in comparison with denervated for some hours. Quadriceps muscle. A and B, same animal. Upper record: muscle denervated 7 days before. Lower: muscle denervated for 76 hours. At the signal of A, 0.6 gm. of caffeine. At the signal of B, 250 micrograms of prostigmine. Between A and B: 19 minutes. C, Upper record: muscle denervated 11 days before; lower: muscle denervated for 78 hours. At the signal: 2 mg. of veratrine. At left of each graph, 80-gm. tension.

nerve) normal muscle is more sensitive to the drug. In the inverse case the denervated muscle becomes the more sensitive.

For testing this hypothesis the quadriceps muscle of one side was denervated 13

days and the homologous contralateral 53 to 126 hours before the experiment. There was, therefore, a very sensitive muscle by denervation, and, as control, the homologous contralateral structure was used in which the function of the motor nerve had been excluded by degeneration (9), but its sensitivity was less than that of the first, because the sensitivity to acetylcholine, at least, begins at the third day of denervation. (9).

In these groups of experiments we also tested veratrine and caffeine—the former, because it has the property of producing impulses of the nerve (10) and of producing muscular contracture (11, 12), and the latter, because it is an important exciting agent at the central nervous system and is able to stimulate the skeletal muscle directly.

The results in table 2 show clearly that the chronically denervated muscle is more sensitive than the normal to prostigmine, veratrine and caffeine (fig. 6).

#### DISCUSSION

*Drugs Acting Directly Upon Smooth Muscle.* Ergotamine, pituitrin, histamine and barium chloride produce a shortening of smooth muscle, regardless of the innervation. This action is a direct one on the muscle fibers (13). Barium chloride can, furthermore, produce some alteration at the myoneural junction (14). The normal uterine muscle is more sensitive to these drugs than the denervated one. Other exceptions to the denervation law may be cited. Rosenblueth (2) pointed out that the normal nictitating membrane is more sensitive to histamine and ergotoxine, and Altamirano, Fernandez and Luco (4) found greater sensitivity to ergotamine and dehydroergotamine in the same normal organ.

*Drugs Acting on the Motor Nerve and on Skeletal Muscle.* Denervated skeletal muscle is more sensitive to prostigmine, veratrine and caffeine. Although it has not been directly demonstrated, our results are in agreement with those of Masland and Wigton (7) because prostigmine can produce stimulation of the nerve, and with those of Arvanitaki and Fessard (10) because veratrine can excite the motor nerve. As far as we know, this property has not previously been described for caffeine. These results, as well as those reported in the literature, reinforce the position of Altamirano, Fernandez and Luco in not accepting Cannon's explanation of the denervation law: that the greater sensitivity of the denervated effector may be due to an increasing permeability of the structure (1). We also agree with them that the formulation of the denervation law must be modified, once the destruction of efferent neurones does not increase irritability to chemical agents but changes this irritability.

#### SUMMARY

The effects of ergotamine, pituitrin, histamine and barium chloride were studied on normal and denervated uterus *in vivo* in cats anesthetized with sodium pentobarbital. Effects of prostigmine, veratrine, and caffeine on normal and denervated skeletal muscles were studied. The normal uterine horn is more sensitive than the denervated muscle to the drugs employed. Quadriceps tibial and soleus muscles crushed immediately before the experiment are more sensitive than the denervated structures to caffeine and veratrine, and are more sensitive to prostigmine in comparison with the

homologous contralateral structure denervated at least 9 days before. If the denervation is older (up to 32 days) the denervated side is the more sensitive.

The sensitivity of the chronically denervated quadriceps muscle is greater to prostigmine, veratrine and caffeine than that of the quadriceps muscle denervated for 13 days with its homologous contralateral crushed centrally for 126 hours.

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# INCREASED RATE OF UREA FORMATION FOLLOWING REMOVAL OF RENAL TISSUE<sup>1</sup>

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THE experiments presented here show that the rate of urea formation is accelerated in rats during the first 24 hours after removal of three quarters or all of the total renal tissue, and that the greater the amount of kidney excised the greater is the quantity of urea formed. The observations were made on rats in which urea formation from food protein was excluded by subsistence on a calorically adequate diet containing no protein. Urea formation was calculated for consecutive 4-hour intervals, the amount of urea formed being derived from changes in the body urea content and from the quantity of urea excreted in the urine.

## METHODS

Three hundred and ninety-two healthy female albino rats, selected at body weights of about 150 gm., were divided into 40 groups of approximately 10 rats each. The average body weight was 150.8 gm. with a standard deviation of 3.6 gm. Before the experiments, all rats were maintained on an adequate stock diet containing 17 per cent protein. After selection, the rats within each group were placed in individual cages and were fed a solution of 15 per cent glucose in water containing 0.4 per cent sodium chloride and vitamins of the B complex. After 48 hours of glucose feeding, 2 groups of unoperated controls were killed, 8 control groups were submitted to a sham operation in which both kidneys were exposed and handled, in 21 groups 75 per cent of the total renal tissue was removed, and in 9 groups a complete nephrectomy was performed. A small amount (less than 1 ml.) of intra-abdominal bleeding occurs from the stump of the remaining kidney when 75 per cent of the total renal tissue is excised. Therefore, one kidney from each rat subjected to total nephrectomy was first incised and allowed to bleed briefly into the abdominal cavity before removal, in order to make the different operative procedures alike except for the amount of renal substance removed. Following operation, the rats were returned to individual cages, and the glucose feeding was continued until autopsy.

Two groups of sham-operated controls were killed at each of the following intervals after operation, at 4, 7, 16 and 24 hours. Three groups of animals from

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which 75 per cent of the total renal tissue had been removed were killed at the end of the 1st, 4th, 7th, 12th, 16th, 20th and 24th hour after operation. Three groups of rats from which all kidney substance had been removed were killed 4, 16 and 24 hours after operation. The rats were killed under ether anesthesia by exsanguination. Aliquot portions of serum were joined to form one serum pool for each group. Urine collections were made immediately before each group was killed, and the individual collections were combined to make one urine pool for each group.

Urea was determined by the urease aeration method of Addis (1), all measurements being made in duplicate. The amount of urea formed during each period was estimated by adding to any urea excreted in the urine during that time the difference between the average urea content of the body found at autopsy and the average urea content of similarly treated groups that had been killed at the end of the preceding period. The urea content of the body was obtained by multiplying the mg. of urea per ml. of serum by the number of ml. of water in the body as calculated from the live body weight, assuming for this purpose that the total body water in a rat is 63 per cent of its body weight. Here we depend on Pace and Rathbun (2) who used the data of Ashworth and Cowgill (3).

Before operation, all the rats were approaching a minimum rate of urea formation. In the groups killed before operation, we found an average serum urea concentration of 9.0 mg/100 ml. Their body weight was 150 gm., and their total water content was estimated as 63 per cent of 150 or 94.5 ml. Their total urea content 48 hours after consuming nothing but glucose was thus  $\frac{9.0 \text{ mg.}}{100 \text{ ml.}} \times 94.5 \text{ ml.}$  or 8.5 mg. of urea. We say they were only approaching a minimum because we have found that when this diet is continued for more than 48 hours somewhat lower serum urea concentrations and lower total body urea contents may be obtained (4). We may assume, therefore, that during the next 24 hours, the period during which our observations were made, urea formation would ordinarily be very slowly falling. Here, then, is a minor error in our calculations, one which has the effect of underestimating the increases in urea formation we found after operation.

It should be noted that the total urea content of each group was estimated at one time only, namely when they were killed. It may seem that we might have obtained more precise results if we had measured the serum urea concentration of each group at the beginning as well as at the end of each period. This, however, would have required tail cutting or cardiac puncture. Our experience has been that either of these procedures may at times, and in certain animals, induce wide deviations from the average behavior of undisturbed controls. Lippman has shown that this is true with respect to renal function in the rat (5). We have no data on the metabolic effects, but the effect of large hemorrhage on the rate of urea formation of nephrectomized rats has been studied by Engel and Engel (6).

The group method we have used requires the use of a large number of rats, nearly 400 in this instance. We have adopted it as preferable to working with individuals, not only because we were thus enabled to avoid bleeding, but also because we believe that when as many as 40 comparable groups are used, we diminish the effect of individual variation. The group of 10 then becomes the individual,

and unexplained fluctuations in behavior are decreased. This, however, is not a random statistical group, but one in which each member of the group is known to have conformed with a series of particular requirements. During the collection of urine, the rat's behavior and appearance had to be not unusual. The urine volume had to be not very much less than that obtained from the others. Finally, at the post-mortem examination made immediately after the urine collection had been completed, it was seen that no urine was left in the bladder, that there were

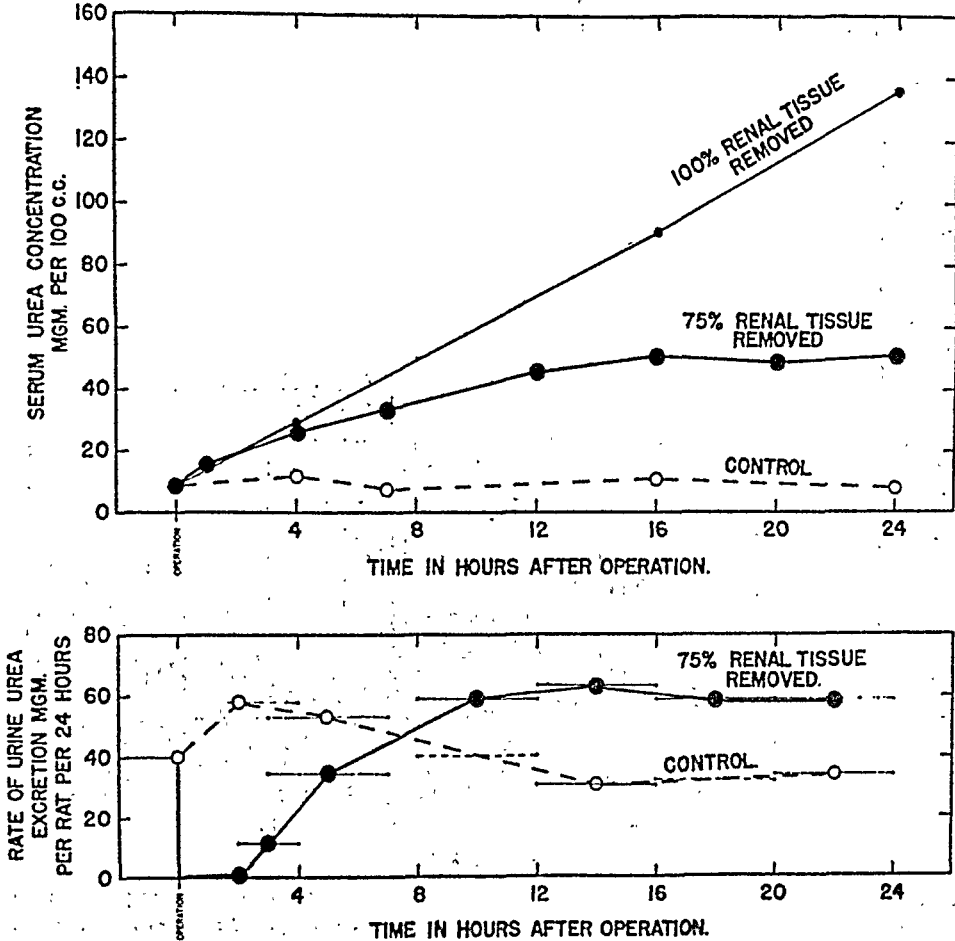


Fig. 1. AVERAGE SERUM urea concentrations and rates of urine urea excretion. The excretory rates are plotted as midpoints and the faint horizontal lines crossing the rates of urea excretion represent the time during which the urine collections were made.

no signs of circulatory failure, no gross organ anomalies, and that the centrifuged blood gave a clot volume neither unusually large or small. When any of these abnormalities were observed the urine and serum of that rat were discarded. Very few of our animals were rejected for any of the above reasons, but that was only because we had the good fortune to work with highly standardized healthy young rats. This is, indeed, a prerequisite for the safe use of such a group method because it is obvious that averages of only 10 concentrations and rates might be misleading if they included even one measurement greatly influenced by some unsuspected factor of an individual nature.

An essential element in the reliability of the results is the degree of precision

achieved in the collection of short time urine collections. The method we used has been described in detail elsewhere (7).

### RESULTS

The serum urea concentrations and the rates of urea excretion for successive 4 hourly periods after operation are shown in figure 1.

In the controls the serum urea concentrations rise a little and the rates of urea excretion increase for the first 2 periods but thereafter both concentrations and rates fall to or below the preoperative levels.

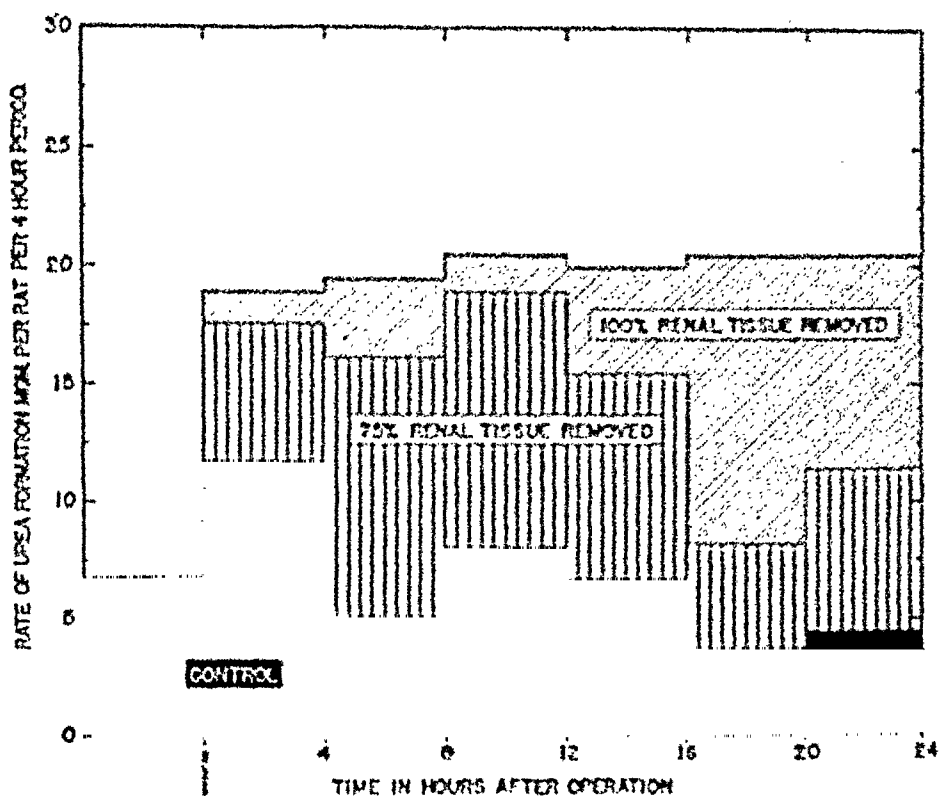


Fig. 2. AVERAGE RATES OF urea formation (urine urea excretion per 4-hour period plus the difference between the body urea content at the beginning and at the end of the period, corrected to 150 gm. body weight).

In the groups from which 75 per cent of the renal tissue was removed the serum urea concentration is almost doubled within an hour after operation and then rises in a straight line for 16 hours and is thereafter maintained at a level four times higher than that of the controls. For the first 2 hours after operation there was complete anuria, and for the second 2 hours the rate of urea excretion was low, but from that time on urea excretion rapidly increased, equalling the control rate by the 7th hour, exceeding it by 100 per cent by the 14th hour and continuing at nearly double the control rate until the 24th postoperative hour.

The completely nephrectomized groups show a steeply rising serum urea concentration that increases steadily until, at the 24th postoperative hour, it is 17 times higher than the concentration of the groups from which 75 per cent of the kidney had been removed.

The amount of urea formed during each postoperative 4-hour period was calculated from the data given in figure 1, by adding to any urea excreted in the urine during that time the difference between the urea content of the body at the beginning and end of the period. The results of these calculations are given in figure 2.

During each successive period the rate of urea formation was greater in the totally nephrectomized groups than in those from which 75 per cent of the renal tissue had been excised, and in turn this latter rate was considerably greater than that of the controls. If we add the total urea formed over the 24 hours of observation we find that the nephrectomized groups formed 120 mg. of urea per rat, the 75 per cent groups 88 mg. per rat and the sham-operated controls 40 mg. per rat.

#### DISCUSSION

The significance of the urea formation figures we have given depends on the validity of the assumptions made in their calculation. It is conceivable that under the particular conditions we observed these assumptions might involve errors so large that they would invalidate our conclusions, not only quantitatively but even qualitatively. For this reason, but mainly because the measurement of the rate of urea formation may prove to be a useful tool in the field of protein metabolism, a discussion of the basis for the method should precede any conclusions.

We are indebted to Engel and Engel (6) for the first clear expression of the idea that we can derive the urea content of the whole body by multiplying the urea concentrations of the serum by an estimate of its total water content. This is possible because urea diffuses into every cell and every fluid of the body, distributing itself through the water of every tissue in equal concentration, a fact first demonstrated by Marshall and Davis in 1914 (8). It follows that if we determine the concentration of urea in 1 ml. of serum and multiply it by the number of ml. of water in the body we can approximate the urea content of the body at that moment. Engel and Engel also saw that if these measurements are repeated after an interval of time we can find to what degree the urea content of the body has increased or decreased. During this interval, urea has been leaving the body through the kidneys, the only place from which urea is excreted at a concentration higher than that in which it exists in the body water. Therefore, if the rate of urea excretion is determined we can get the amount of urea that is formed by adding to the excretion rate the increase or subtracting from it the decrease in the urea content of the body that has occurred during the time interval over which the rate is measured.

Admittedly the calculation can give us no more than a first approximation<sup>2</sup> because the water content of the body is a quantity so hard to define. There is water that for many metabolic purposes is outside of the body, which is within it as far as urea is concerned. Urea diffuses into the large and variable volume of water that comes and goes between the body and the interior of the gastrointestinal tract. Not all of the urea that enters the gut returns as such, for part or

<sup>2</sup> The degree to which urea concentrations may deviate from an exact equality of concentration in the water of each part of the body is not yet decided. See, for instance, the careful work of Ralls (9).

all of it may be decomposed to ammonium carbonate by urease containing bacteria in the colon. This, of course, is no final loss because the liver will form urea again when the ammonia comes to it in the portal blood. However, the facility with which urea diffuses into the alimentary canal makes it necessary to take the water in the alimentary canal into account and this is done when we base our estimate of total body water on live body weight.

In our particular experiments there is a special criticism arising from the fact that we did not measure the body water but used an average calculated estimate. For preliminary purposes of relative orientation this may be permissible when the conditions are such that we need not suspect rapid changes in total body water. But in our experiments we had reason to think that the water content of the completely nephrectomized rats had increased. The tissues at the end of the 24-hour period seemed to be wetter than usual and there was some free abdominal fluid not found in the other groups. From an absolute point of view we therefore cannot place any reliance on the precision of the 120 mg. per rat urea formation we found over the 24 hours in the totally nephrectomized groups. But it is important to notice that the error is such that it would lead to an underestimate of the actual amount and that, *a fortiori*, the qualitative conclusion that there is an increase in urea formation in nephrectomized rats still stands. A constant error is, of course, incurred by treating serum as if it were water.

But to us the most important observation that can be made about the calculation of urea formation is that we can only hope for unambiguous results when the measurements are made under the conditions we used with respect to the removal of food protein from the gastro-intestinal tract. The production and absorption of amino acids in the gut is so variable and, under ordinary circumstances, so large, that unless this factor is eliminated it is difficult to assign any precise meaning to urea formation results. Only when we can be sure that none of the urea that is formed comes from precursors external to the body can we take the figures as an index of the rate of body protein catabolism. That, even then, it is a complete measure of body protein catabolism is still open to question, but that it measures a preponderating part of the total catabolism is at least a reasonable supposition. This, however, is no more than an addendum to the theoretical considerations presented by Ogden and Tripp (10).

If we accept our results as indicating, not quantitatively but qualitatively, that the more renal tissue we remove the greater is the rate of urea formation, we have to ask what reason there may be for this, to us, surprising finding. It is not an isolated observation, for several years ago it was shown in this laboratory that the rate of urea excretion was considerably increased in quarter kidney rats fed only glucose, an increase that was accompanied by an increase in serum urea concentration, so that there must have been an increase in urea formation (11). We cite these experiments because they extended over a period of 7 days. It would seem then that what we observe in the present experiments within a space of 24 hours, is more than a transient postoperative phenomenon. Yet it is contrary to the conclusions of Reid (12) who finds a decrease in urea formation in nephrec-

tomized rats which he attributes to the cessation of deamination in the kidney. Further, Mylon, Smith and Goldstein (13) show a decrease in urea formation in dogs with nitrogen retention induced by reduction in the quantity of renal tissue when amino acids are given. It may be that the increase in urea formation we find occurs only when the rate of protein metabolism has been reduced to a minimum before operation by giving no protein and a calorically adequate amount of glucose. In the present experiments we were not able to get precise measurements of the amounts of glucose consumed. We suspect that the variations in the formation of urea shown in figure 2 during the successive periods, particularly evident in the controls, may have arisen because many of the collections were made during the day when rats go to sleep and drink less than in the night. Even transient caloric inadequacy may raise the level of urea formation when protein metabolism is very low. For in experiments we shall report later we observed an increase within four hours after no food was given and this was more marked where water as well as glucose was withheld.

The first explanation of our findings that comes to mind is that the 3 degrees of urea formation are a consequence of three degrees of trauma in the operations. To us this seems inapplicable because in all cases a double laparotomy was performed, in all cases the kidneys were handled and in no case did any operation take more than a few minutes. It is true that of the 3, the 75 per cent removal was presumably the most traumatic, since half of the remaining kidney was excised. Yet in the simpler operation of double nephrectomy we find a greater increase in urea formation.

When Bondy and Engel (14) nephrectomized rats they found that the rate of urea formation rose until they died, but when the adrenals as well as the kidneys were removed the level of urea formation did not change. The simplest explanation of our results would be to suppose that some substance that accelerated protein metabolism was retained in the body as a consequence of the removal of renal tissue. That cannot be the only factor because the increase in urea formation occurs during the first four hours after operation, and has no relation to the degree of retention of urea. But it is still possible to view the total increase as the combination of an initial increase due to trauma, plus a later increase due to uremia. We adopt this as a provisional working hypothesis.

#### SUMMARY

On a diet of 15 per cent glucose in 0.4 per cent sodium chloride when urea excretion is approaching a minimum, the rate of urea formation in rats is increased during each 4-hour period of the first 24 hours after removal of 75 per cent of the total renal tissue, the increase being measured relatively to similarly traumatized control rats whose kidneys were left intact. Under the same conditions, a greater increase in urea formation follows the complete removal of both kidneys. During the first 24 hours after operation, controls with both kidneys intact form 40 mg. of urea per rat per 150 gm. body weight, rats left with a quarter of their renal tissue form 88 mg. and rats with no renal tissue produce 120 mg. of urea.

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Inflation of the intracranial balloon was followed by respiratory depression and sometimes apnea in addition to the above-mentioned effects. Enough fluid (average 5.8 cc.) was injected into the balloon to produce bradycardia, but intracranial tension was not maintained at a level sufficiently high to cause marked respiratory depression. In certain animals the range between maintained bradycardia and severe respiratory depression was quite narrow, and in these cases it was difficult to establish a persistent large increase in mean pulmonary venous pressure. However, since the oxygen content of femoral arterial blood fell on the average only 1.4 vol. per cent after brain compression, it seems unlikely that hypoxemia played a major rôle in these experiments. The possibility has not been ruled out, however.

On the basis of earlier work on guinea pigs and because of the corroborative evidence supplied by the development of bradycardia and high pulmonary venous pressures in the dogs subjected to increased intracranial pressure a working hypothesis may be advanced. The hypothesis is that brain compression is associated with increased vagal tone, and the latter results in bradycardia, high pulmonary vascular pressures, lowered cardiac output and pulmonary lesions. Restoration of the heart rate to control levels or higher with atropine resulted in the reestablishment of normal cardiac outputs and in the return to normal of mean pulmonary vein and artery pressures. Subsequent additional increases in the elevated intracranial tension were without effect in the atropinized animals. The 4 atropinized dogs showed only slight pulmonary pathology at autopsy, and 3 of these animals were killed while in good condition. On the basis of these findings one might suggest that the administration of atropine to patients *exhibiting bradycardia* as a result of increased intracranial pressure following traumatic or spontaneous intracranial hemorrhage might be tested as a measure which might decrease the incidence or severity of the lung edema otherwise seen frequently.

#### CONCLUSIONS

Elevation of the intracranial pressure in anesthetized dogs was found to be followed ordinarily by *a)* bradycardia, *b)* lowered cardiac output and *c)* increased mean pulmonary venous and arterial pressures. These effects were independent of whether there was spontaneous or artificial respiration. When the pulmonary venous pressure rose above 20 mm. Hg significant degrees of pulmonary edema, congestion and hemorrhage were seen at necropsy. In some instances such lesions were seen at lower venous pressures. Administration of atropine after induction of the changes listed above as following maintained elevation of the intracranial pressure, reversed those changes, and significant pulmonary edema did not occur.

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# CIRCULATORY CHANGES IN THE DOG PRODUCED BY ACUTE ARTERIOVENOUS FISTULA<sup>1</sup>

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CIRCULATORY changes produced by an arteriovenous fistula have been extensively studied. Conflicting ideas however still persist (1-6). The present study was undertaken to see whether or not some of these contradictions might be resolved by the simultaneous and systematic determination of a number of these factors. Measurements of systemic and pulmonary arterial pressures, central venous pressure, heart rate, cardiac output, flow through several parts of the body and local peripheral resistance were made in order to compare the immediate effects of opening and closing the fistula on circulatory dynamics.

## METHODS AND RESULTS

*General.* Twenty dogs weighing 11 to 22.5 kg. were used. Nineteen of the animals were anesthetized intravenously either by chloralose (100 mg/kg.), chloralose plus morphine (0.5 mg/kg.), urethane (1.5 mg/kg.) plus morphine, or sodium pentobarbital (25 mg/kg.) (table 1). In addition, one unanesthetized trained dog with an A-V fistula of four weeks' duration was studied. In 12 dogs A-V shunts about 2 cm. in length were produced between the left femoral artery and vein approximately 5 cm. below the inguinal ligament by means of a side to side anastomosis in the manner described by Holman (3). In the remaining 8 dogs, a paraffinized glass U-tube was connected to the proximal ends of the left femoral artery and vein: 30 to 50 mg. of heparin being injected intravenously to prevent clotting in the cannula. Our main interest was the study of the general circulatory changes rather than those occurring locally.

*Blood Pressure and Heart Rate.* Right femoral arterial pressure and heart rate were determined in each dog from a Hamilton manometer record. All measurements were made about 15 minutes after the shunt was first established. Pressures and rates recorded continuously during the periods with the fistula open, with the fistula closed for one minute, and with the fistula reopened for one minute, are tabulated in table 1. Compression of the fistula consistently produces a sudden increase in both systolic and diastolic pressure which reaches a peak in 3 seconds and is usually associated with a slowing of the rate (Nicoladoni-Branham sign). The blood pressure then falls somewhat and becomes stabilized at a level somewhat higher than that found

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TABLE 1. BLOOD PRESSURE AND HEART RATE CHANGES OBTAINED UPON COMPRESSION AND REOPENING OF THE FISTULA

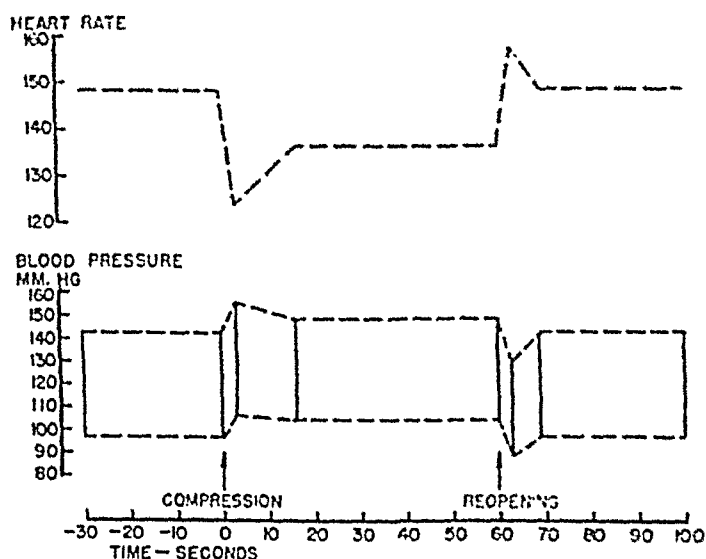


FIG. 1. THE AVERAGE PRESSURE and heart rate response in the 10 animals anesthetized with chloralose.

prior to compression. With release of compression, the picture is reversed. There is a drop in blood pressure to the lowest level at the end of 3 seconds and an increase in heart rate with a return to the original values. The average pressure responses and rate changes in the 10 dogs anesthetized with chloralose can be seen in figure 1.

Between the peak and the stabilization of the pressure curve, a 'rebound effect' is frequently found during which the pressure may drop almost as low as the level prior

to compression. The same phenomenon but in the opposite direction is noted upon release of the fistula. This type of curve, illustrated in figure 2, was present in *dogs* 3, 4, 5, 7, 13 and in all dogs anesthetized with sodium pentobarbital.

*Pressures in the Vessels at the Site of the Fistula.* In 7 dogs, pressures were obtained in the femoral vessels distal and proximal to the fistula and in the fistula itself.

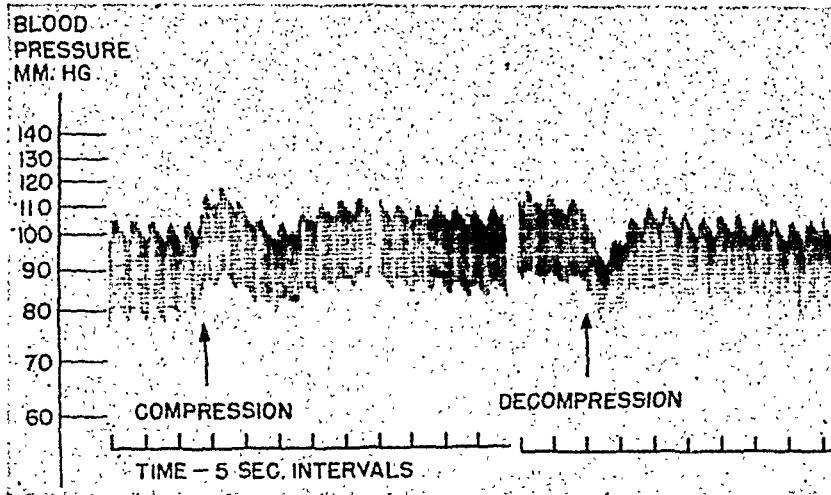


Fig. 2. PRESSURE CURVE illustrating the 'rebound effect'

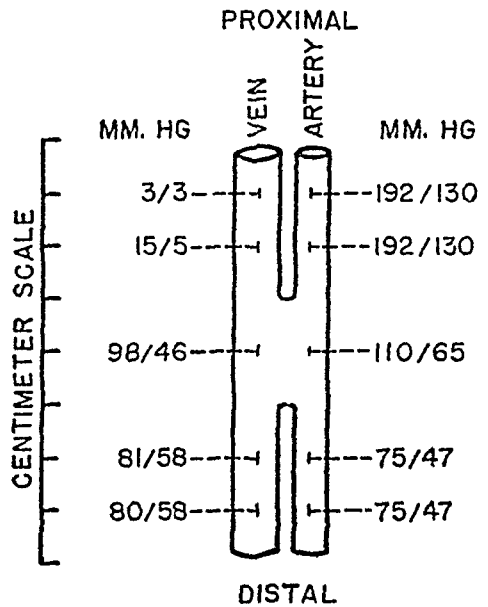


Fig. 3. TYPICAL PRESSURE READINGS in and around an arteriovenous fistula.

A typical example of the results is shown in figure 3. It can be seen that the pressure in the proximal artery is maintained without significant change almost to the site of the fistula. It falls sharply in the fistula proper and in the proximal vein where the pulse pressure becomes zero at a distance of 2 cms. from the fistula. In 3 cases, the pressure in the distal vein was slightly higher than in the distal artery and occasionally a slow retrograde flow into the distal vein was seen.

*Blood Flow in Various Parts of the Body.* The fact that the blood pressure and heart rate changes are not maintained at the maximum levels on opening and closing

the fistula indicates that certain compensatory mechanisms are called into play. One of the compensatory factors could be a change in blood flow to other parts of the body. To check this possible factor a Ludwig strommuhr was introduced into the opposite femoral artery and blood flow measurements were made with the fistula open and closed. Figure 4 shows typical changes in flow correlated with the mean arterial pressure. Some parallelism is present between the two. Nevertheless the results shown in table 2 suggested that the marked differences in blood flow are not entirely

Fig. 4. A TYPICAL CURVE showing the changes in flow observed in the contralateral femoral artery, correlated with the mean arterial blood pressure.

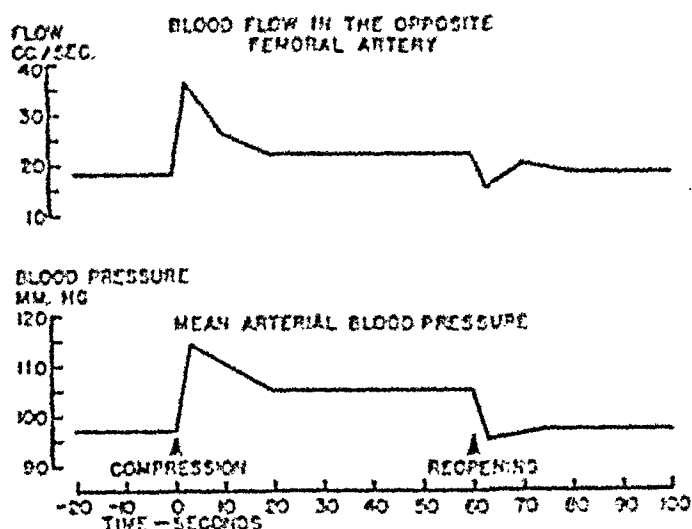


TABLE 2. EFFECT OF OPENING AND CLOSING A-V FISTULA UPON CONTRALATERAL FEMORAL ARTERY FLOW

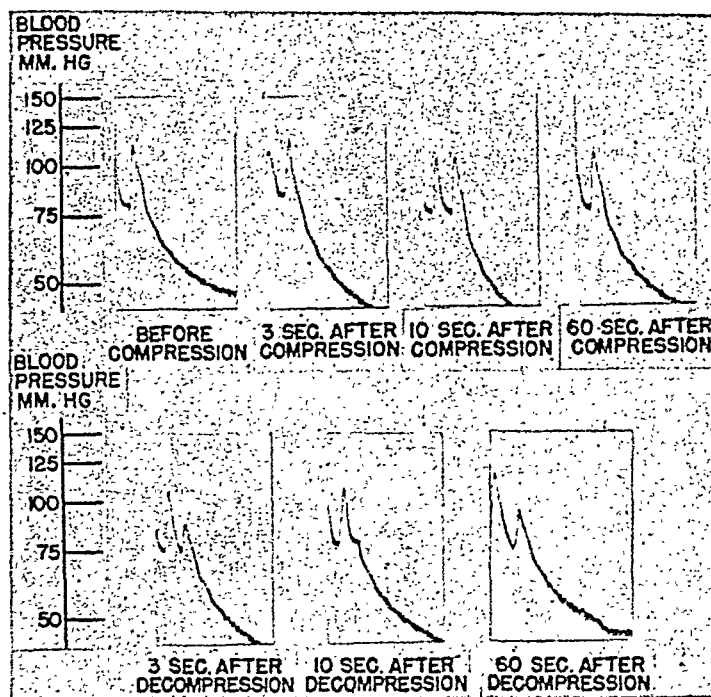
DOC. NO.	FISTULA CLOSED	FISTULA OPEN	DIFFERENCE
	cc/min.	cc/min.	%
3	20	18	-38
4	100	60	-40
5	52	36	-31
6	48	28	-42
7	22	18	-18
8	34	27	-21
Average.....	47	31	-34

due to the changes in mean arterial pressure but are due in part to some reflex vasomotor changes in the blood vessels. To investigate the presence of active vasomotor responses to the fistula, measurements of *asystolic* blood pressure gradients in the opposite femoral artery were made. These measurements were performed in dogs 2, 3, 4, 5, 8 and 10 using the technique described by Williams and Schroeder (7). This technique is based upon the principle that when an artery is suddenly clamped, the gradient of pressure fall distal to the clamp is a function of the degree of peripheral resistance in this artery. Numerous readings were taken with the fistula open and closed. A typical example of these gradients is shown in figure 5. It can be noted that within 3 seconds following compression of the fistula, the peripheral resistance

has markedly decreased and after 10 seconds when the blood pressure has already fallen, the peripheral resistance is apparently decreased further. This indicates an active vasodilatation at 10 seconds. The opposite effect occurs on reopening the fistula indicating an active vasoconstriction.

The possibility exists that changes in blood flow occur in other vessels of the body as well as in the opposite femoral artery. This was studied further by means of venous oxygen deficit determinations. Since the total metabolism of the body remains unchanged when the fistula is open and closed (see below), it was postulated that within the times required for these experiments, no changes occur in the local tissue metabolism. Therefore, the comparison of oxygen deficits in various veins of the body should give a good indication of the changes in flow from the areas drained

Fig. 5. SYSTOLIC BLOOD PRESSURE gradients obtained upon opening and closing the fistula.



by these veins. Blood samples were taken in the usual way before compression and after one minute of compression and oxygen content determined with the usual Van Slyke technique. Percentage change in flow was calculated according to the following formula:

$$\% \text{ change in flow} = \frac{AV_0 \times 100}{AV_c} - 100$$

where  $AV_0$  = arteriovenous oxygen difference with fistula open, and  $AV_c$  = arteriovenous oxygen difference with fistula closed.

Table 3 shows that there is a decreased blood flow in the opposite femoral vein, the jugular vein and the superior vena cava. The opening of the fistula therefore does lead to a decrease in flow in other parts of the body. An increased flow was found in the inferior vena cava in a number of experiments. It is explained by the increased amount of blood being shunted through the fistula. In *dog 18*, there was no such increase in inferior vena cava flow indicating that on some occasions the blood

TABLE 3. CHANGES IN BLOOD FLOW WITH THE FISTULA OPEN

DOG NO.	% CHANGE IN FLOW			
	Femoral vein	Jugular vein	Superior vena cava	Inferior vena cava
4	-40			
5	-33			
6		-9		
7		-17		
8	-45	-14		
10	-17	-18	-20	+34
12	-13	-26	-21	+33
13	-8	-26	-10	+18
14	-21	-15	-20	+12
15	-18	-22	-20	+45
18	-15	-21	-3	0

TABLE 4. CARDIAC OUTPUT MEASUREMENTS

DOG NO.	ANESTHESIA	WEIGHT, KG.	FISTULA OPEN				FISTULA CLOSED				DIFFERENCE	
			Heart Rate	O <sub>2</sub>	AV	CO	Heart Rate	O <sub>2</sub>	AV	CO	Heart rate	CO
9	Chl Mor	16.5	141	93	3.08	3.34	130	93	4.34	2.14	7	9
after 2 mg. atrop.			203	121	3.88	3.12	203	121	4.00	3.02	0	3.3
10	Chl Mor	11	163	85	6.04	1.28	160	85	8.50	0.00	2	19
12	Ureth Mor	22.5	105	100	4.24	2.30	97	100	4.00	2.12	8	11
13	Ureth Mor	13.5	154	83	3.28	2.53	143	83	3.73	2.23	8	15
after 2 mg. atrop.			220	100	2.30	4.35	220	100	2.30	4.35	0	0
14	Ureth Mor	11	149	65	2.10	2.97	143	65	2.31	2.81	4	5.6
after 1.5 mg. atrop.			178	68	2.06	3.30	178	68	2.06	3.30	0	0
15	Ureth Mor	11.5	136	58	2.33	2.49	127	58	2.81	2.06	8	21
after 1.5 mg. atrop.			154	67	2.00	3.20	149	67	2.21	3.03	3.3	5.5
18	Pent	15.5	126	90	4.70	1.91	126	90	4.70	1.91	0	0
19	Pent	14	230	86	4.10	2.10	228	86	4.22	2.04	0.8	3

O<sub>2</sub> = Oxygen consumption in cc. per min. AV = Arteriovenous difference in vol. oxygen %.  
 CO = Cardiac output in liters per min. Chl Mor = Chloralose—Morphine. Atrop = Atropine.  
 Pent = Pentobarbital Na. Ureth Mor = Urethane—Morphine.

flow from veins draining the lower part of the body is reduced sufficiently to neutralize the increase in flow through the fistula.

*Central Venous Pressures.* Since it was found that in most cases of acute arteriovenous fistula there was an increased blood flow in the inferior vena cava, it was considered worthwhile to determine whether there was an associated change of venous pressure in this vessel. Measurements were therefore made by catheterizing the inferior vena cava near the heart via the right jugular vein. At first a saline manometer was used but the readings proved unreliable. The same measurements were then repeated with a very sensitive Hamilton manometer. In no instance was an increase in central venous pressure found on opening the fistula despite the increase in rate of blood flow.

TABLE 5. COMPARISON OF CHANGE IN CARDIAC OUTPUT ON OPENING A-V FISTULA WITH FLOW THROUGH FISTULA

DOG NO.	CARDIAC OUTPUT FISTULA OPEN	CARDIAC OUTPUT FISTULA CLOSED	DIFFERENCE	FLOW THROUGH FISTULA
	l/min.	l/min.	cc/min.	cc/min.
19	2.10	2.04	60	160
9 after atropine	2.34	2.14	200	320
	3.12	3.02	100	320
10	1.28	0.99	290	320
12	2.36	2.12	240	450
15	2.49	2.06	430	480
13 after atropine	2.53	2.23	300	440
	4.35	4.35	0	440
14 after atropine	2.97	2.81	160	200
	3.30	3.30	0	200
18	1.91	1.91	0	160

*Cardiac Output Studies.* Cardiac outputs were measured in 8 dogs by the direct Fick method. Arterial blood was obtained from the right femoral artery. Mixed venous blood was drawn from the pulmonary artery by means of a catheter introduced through the jugular vein under fluoroscopic control. The oxygen consumption was determined by means of a basal metabolism apparatus attached to a tracheal cannula. All blood samples were taken after the fistula was opened or closed for one minute. The results of these studies are detailed in table 4.

In 8 dogs the blood flow through the fistula was correlated with the cardiac output.

The oxygen consumption in each instance was unchanged by opening or closing the fistula. These were the animals in which a glass cannula was used to produce an arteriovenous shunt. Approximate values for the blood flow were obtained at the end of each experiment by detaching the cannula from the femoral vein and measuring the volume of blood loss during 10 second intervals. Table 5 shows the results ob-

tained. In no instance did the increase in cardiac output compensate entirely for the amount of blood flow through the shunt, confirming again the observation that opposite changes in blood flow occur in other parts of the systemic circulatory system.

*Pulmonary Artery Pressures.* Pulmonary artery pressures were measured in 6 animals, with a sensitive Hamilton manometer (table 6). A typical example of the pressure changes in the pulmonary artery pressure is shown in figure 6.

TABLE 6. EFFECT OF A-V FISTULA UPON THE PULMONARY ARTERIAL PRESSURE

DOG NO.	SYSTOLIC PRESSURE	DIASTOLIC PRESSURE	CARDIAC OUTPUT (L. PER MIN.)
	mm. Hg	mm. Hg	
6	+2	+2	
10	+3.2	+3	+29
12	+1.3	+1	+21
14	+1.5	+1	+5.6
after atropine	0	0	0
15	+2	+1	+21
after atropine	+1	+0.7	+5.5
18	+1	+0.5	0

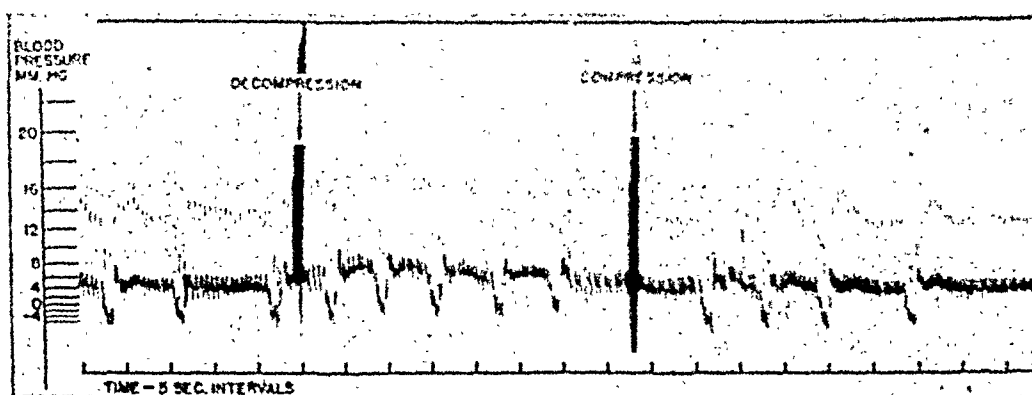


Fig. 6. TYPICAL CHANGES in pulmonary artery pressure on closing and opening the fistula

#### DISCUSSION

The choice of a suitable anesthetic agent is essential for the proper study and evaluation of the hemodynamic changes in heart rate and cardiac output. Because of its inhibiting action on the vagi, pentobarbital anesthesia cannot be used in experiments which require normal vagal responses. Studies on heart rate and cardiac output in unanesthetized trained dogs (unpublished data) have shown that the closest approximation to normal conditions was obtained by the use of chloralose anesthesia. Under urethane anesthesia, vagal responses were adequate for the purposes of this investigation.

Femoral fistulae were preferred because they were far from the heart and large shunts could be constructed easily in this area. Jugular-carotid anastomoses were not used because of their proximity to the carotid sinus.

From the data obtained it appears that there are two mechanisms by which the cardiovascular system immediately compensates for the flow of blood through the fistula; a decreased blood supply to various parts of the body and an increased cardiac output. The decrease in blood flow in other vascular areas when the fistula is open is due not only to a lower mean arterial pressure but also in large measure to peripheral vasoconstriction. Following the establishment of the fistula the cardiac output is increased.

The fact that the central venous pressure does not change at any time is significant. The explanation for the absence of any change in central venous pressure is as follows: in the 3 to 5 seconds following the opening of the fistula the arterial pressure is decreased causing a fall in the head of pressure on the venous side. After this period of time the peripheral vasoconstriction and the increased cardiac output come into play. Associated with these changes there is also an increased velocity of blood in the inferior vena cava as shown by Heringman, Davis and Rives (8). The peripheral vasoconstriction reduces the returning venous flow from the periphery outside of the fistula circuit. Despite the reduction of peripheral venous flow there is an increase in central venous flow coming from the fistula which is absorbed by the increase in cardiac output without any central venous pressure rise.

On the basis of these findings it is felt that this dual mechanism does not originate on the venous side. Since the central venous pressure does not increase, the Bainbridge reflex can be eliminated as a factor in the compensatory changes. These are produced by increased sympathetic tonus and decreased vagal tonus, probably carotid sinus and cardio-aortic in origin. Once these factors are initiated, the increased cardiac output is maintained by the increased flow in the inferior vena cava and the resultant improved filling of the heart. The increased returning flow also contributes to a large stroke volume.

#### SUMMARY

Acute circulatory changes were studied in 20 dogs after the introduction of an arteriovenous fistula between the right femoral artery and vein. Different anesthetic agents were used and their importance discussed. Studies of the dynamics of circulation indicate that the following changes occur when the fistula is open: *a*) there is a reduction of the systolic, diastolic and mean arterial pressure; *b*) the heart rate is faster; *c*) there is a vasoconstriction in the periphery outside of the fistula circuit and the returning venous flow from these areas is decreased; *d*) the central venous pressure remains unchanged; and *e*) cardiac output and stroke volume increase.

Compensation for the increased flow of blood through the fistula takes place by the increased cardiac output and the decreased flow of blood from areas outside the fistula circuit.

We are indebted to Dr. L. N. Katz for his guidance and constructive criticisms during the course of this study.

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# EFFECTS OF GRADED PRESSURES ON THE TAIL OF THE MOUSE<sup>1</sup>

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REVIEW of the literature reveals that few studies have been made of the effects of known pressures applied to limited areas of the body's surface. That these effects are important, however, is shown not only by the *a priori* recognition that they may give added insight into the mechanisms which transport substances to and from the immediate vicinity of the cells but also by the wide use of therapeutic pressure dressings. Brooks and Duncan have devised a method for applying pressure uniformly to the tail of the rat by a pneumatic sheath (1). They, however, made only limited studies of the lower range of pressures. Their method, moreover, involves the close confinement of the animal, which would prevent it from feeding and exercising and thus limit the time during which pressure could be applied continuously.

The present study was devoted chiefly to the effects of the infrasystolic range of pressures. The necessity for the close confinement of the animal was avoided by developing a pneumatic sheath so light and compact that it could be applied to the tail of a mouse without interfering seriously with its ability to feed and exercise.

## GENERAL PROCEDURE

The apparatus is shown and explained in figure 1. Adult, inbred mice (strain Strong A) of both sexes were used. They were kept in cages with a litter of shavings and given Purina Fox Chow and tapwater *ad libitum*. The laboratory temperature varied somewhat, but this did not seem to affect the result, for the variation in experiments run in parallel was as great as in experiments run serially. It was necessary to remove the points of the lower incisors to prevent the animals from gnawing the equipment, and in the longer experiments this may have interfered with nutrition. A tattoo mark was placed on the dorsum of each animal's tail near the base to serve as a reference point for measuring.

## EXPERIMENTS

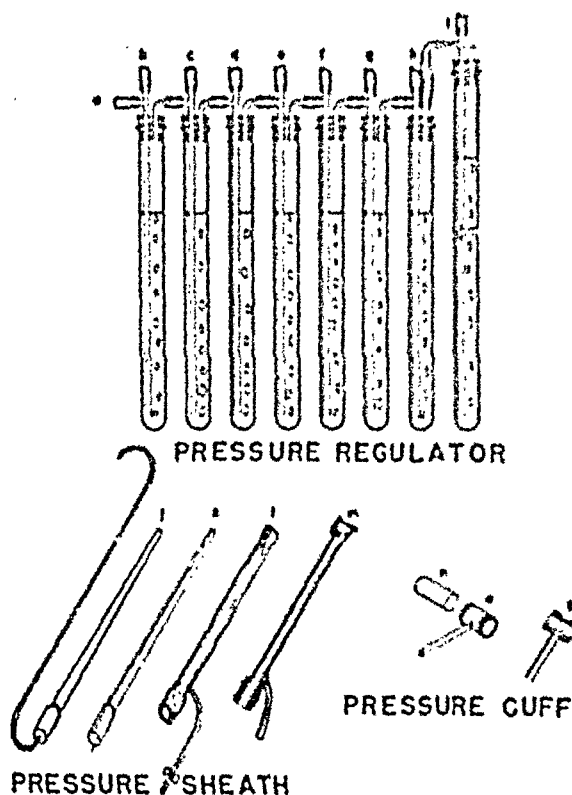
*In the first group of experiments, a study was made of the length of necrosis present a week after known pressures had been applied to the tail for known intervals by means of the pneumatic sheath. The results are shown in table 1. A pressure of 30 mm. Hg applied for as long as a week did not cause obvious inflammation or necrosis. A pressure of 40 mm. Hg for 4 days or longer resulted in some inflammation and a little necrosis at the tip. Higher pressures caused the necrosis to extend farther proximally. Between 70 and 80 mm. Hg pressure there was marked increase in the length of necrosis. At 100 mm., only a short length of the tail within the sheath*

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survived. Uncertainty concerning the accuracy of the transmission of pressure at the proximal end of the sheath discouraged study of the 110- and 120-mm. pressures. A pressure of 140 mm., which presumably allowed no blood to enter the tail, produced



**FIG. 1. APPARATUS.** The pressure regulator consists of 8 glass tubes 30 mm. in diameter. Seven are 40 cm. long and 1 is 50 cm. long. 'a' leads to a source of compressed air. By adjustment of the water levels, a regular series of pressures is available at 'b-i'. Intervening pressures can be had by varying the depth of insertion of the small tube at 'f'. The flow of air should be regulated so that some is always escaping at the far end. This system avoids the poisonous fumes of the usual mercury pressure regulator, and gives more even pressures.

The liner, 'k', of the pressure sheath made of gum rubber, which must be so thin that only a couple millimeters of mercury pressure are needed to collapse it. The stainless steel mold, 'j', is coated with a suitable coagulant and dipped in 20% natural latex. It is removed briefly and the fine Nylon thread is gently laid in place. It is then redipped to attach the thread. Next the coagulant is washed out and the rubber is dried and cured. The exact details depend on the latex used and must be learned by experience. (The latex and coagulant were furnished by American Anode.) The finished liner is coated with talc. The tapered part of the mold must be somewhat larger than

the tails of the mice to be studied. The outer case is a length of polyvinyl chloride plastic tubing ( $\frac{1}{8}$ " bore,  $\frac{1}{4}$ " wall). A small inlet tube of the same material is sealed on with a solution of the plastic (Tygon Paint) and a collar fashioned from bronze shim stock is inserted into the near end. The sheath is inserted and the segment of larger diameter is turned back over the case and cemented in place with flexible collodion. A thread which had previously been tied to the tip of the liner is now drawn taut and secured by forcing a cork into the far end of the case. This cork is sealed in with flexible collodion to prevent it from working out and to insure an airtight joint. A wire fitting with 2 loops is fastened to the inlet tube so that it will lie over the lumbar region of the mouse when the sheath is placed on the tail. Inflation of the sheath to pressures of 20 mm. Hg or more causes it to grip the tail securely. The wire loops on the inlet tube are sutured to the skin over the lumbar region to protect the tail from any stress from the inlet tube as the mouse moves about in its cage. The inlet tube must be long and slender so that it can be twisted considerably without kinking. A manometer is connected into the tube leading from the pressure regulator to the inlet tube to check the pressure. The sheath is tested for leaks by clamping the inlet tube. If it does not relax its grip on the tail within a few minutes, it may be considered airtight.

The pressure cuff is made of a piece of brass tubing 8 mm. long with a 5.2 mm. inside diameter. The thin rubber liner is made from natural latex by dipping a suitable mold. It is fastened in place by thread and flexible collodion.

an inflammatory response when applied 6 hours, some necrosis of the tip when applied 12 hours, and necrosis of all the tail subjected to pressure when applied 18 hours.

In the second group, a study was made of the transport of fluorescein into and out of the tail during the application of pressure (see table 2). Pressure was applied as before. After 10 minutes, 0.2 cc. of 2 per cent sodium fluorescein was given intra-

TABLE 1. NECROSIS OF DISTAL END OF TAIL FOLLOWING GRADED PRESSURES

PRES- SURE	DURATION OF PRESSURE	LENGTH OF DISTAL END NECROTIC AFTER A WEEK	AVERAGE NECROSIS, FOR 48 HOUR GROUP	REMARKS
<i>mm. Hg</i>	<i>hrs.</i>	<i>cm.</i>	<i>cm.</i>	
30	48	0.0, 0.0, 0.0, 0.0	0.0	Tissues in good condi- tion, some traumatic ulcers at margin of sheath
30	72	0.0		
30	96	0.0, 0.0		
30	120	0.0, 0.0		
30	144	0.0, 0.0		
30	168	0.0		
40	48	0.0, 0.0, 0.0, 0.0	0.0	Some inflammation
40	72	0.0		
40	96	0.0, 0.05		
40	120	0.01		
40	132	0.25		
40	168	0.0, 0.01		
50	48	0.1, 0.1, 0.1, 0.1, 0.25, 0.25	0.15	Surviving tissues in good condition
60	48	0.25, 0.75, 0.75, 1.0, 1.25, 1.5, 1.5	1.0	
70	48	0.3, 0.5, 0.5, 0.5, 1.5, 1.5, 1.5	0.9	
80	48	1.75, 2.0, 5.0, 6.0, 6.25, 7.0, 8.5	5.2	Surviving tissue chiefly ventral and in poor condition
90	48	4.0, 6.0, 6.5, 7.0, 7.0, 8.5	6.5	
100	48	7.0, 7.5, 7.75, 8.0, 8.25, 8.5	7.85	
140	6	0.0, 0.0, 0.0, 0.0, 0.0, 0.01		Temporary inflamma- tion, fading within 7 days
140	12	0.2, 0.6		Tail red, shiny, and con- tracted after 7 days
140	18	8.4, 8.5		

Tails were inserted into sheaths 8.5 cm.

TABLE 2. TRANSPORT OF FLUORESCEIN DURING THE APPLICATION OF PRESSURE

PRESSURE	LENGTH OF DISTAL END INTO WHICH FLUORESCEIN DID NOT PENETRATE IN 15 MIN.	LENGTH OF DISTAL END FROM WHICH FLUORESCEIN WAS NOT REMOVED IN 24 HRS.	LENGTH OF DISTAL END NECROTIC AFTER A WK.
<i>mm. Hg</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>
40		0.0	0.0
40		0.0	0.0
50		.2	.3
50		.2	.2
70	1.75	1.0	1.5
70	0.5	1.5	1.25
80	2.0	5.0	5.0
80	0.5	.5	1.0
80	1.5	5.5	6.0
80	3.0	4.5	5.0
90	3.0	8.0	7.7
90	0.75	4.0	4.25
90	0.0	8.0	8.0
90	0.75	7.0	6.0
100	0.0	7.0	7.0
100	3.0	4.5	6.5

Tails were inserted into sheaths 8 cm.

Pressure was applied for 24 hours.

peritoneally (2). After 15 minutes more, the circulation through the tail was completely stopped by inflating the pressure cuff which had been placed proximally to the sheath. The sheath was then removed, and the distance to which the fluorescein had penetrated was revealed by illuminating the tail with Woods' Light. It was found in some instances that the fluorescein was present, at least in the vicinity of the larger vessels, well within the region that later became necrotic after the tail had been subjected to the same pressure for 24 hours. Next the cuff was removed, and the circulation left unimpeded for 15 minutes, during which the entire tail became brightly fluorescent. The sheath was then reapplied for 24 hours. At the end of this period the fluorescein had been removed from the skin to roughly the same distance that the tail remained non-necrotic throughout the subsequent week.

*In the third group, a study was made of the movement of fluorescein into and out of the tail after a period of pressure.* Pressure was applied for 48 hours, and fluorescein was given 10 minutes before it was discontinued. The tails had become diffusely fluorescent 2 hours after the release of pressure somewhat distal to the point to which

TABLE 3. REMOVAL OF FLUORESCIN AFTER THE RELEASE OF PRESSURE

PRESSURE	TAIL IN BREATH	DISTAL END FROM WHICH FLUORESCIN WAS NOT REMOVED IN 6 HRS.	DISTAL END NECROTIC AFTER A WEEK
mm. Hg	cm.	cm.	cm.
70	7.25	1.25	1.25
70	8.75	1.5	1.5
80	7.75	3.5	3.5
80	8.00	3.5	3.5

Pressure was applied 48 hours.

fluorescein had disappeared 4 hours later. The intervening segment of skin remained fluorescent for over 24 hours. Its proximal margin marked the border of necrosis apparent a week later (see table 3).

*In the fourth group, an approximate, systolic pressure was estimated.* The pressure cuff was applied to the base of the tail and inflated to a suprasystolic level. The mouse was then given fluorescein and illuminated with Woods' Light. After 10 to 15 minutes the pressure in the cuff was reduced 5 mm. at 5-minute intervals. The pressure at which the first trace of fluorescence was detected beyond the cuff was taken as the systolic pressure. The value observed varied with the width of cuff used, being greater for the narrower cuff. It also averaged higher than the value obtained by a plethysmographic method in which the same width of cuff was used (3).

#### DISCUSSION

The finding that a pressure of 30 mm. Hg did not cause any apparent damage even if applied for a week probably means that vasodilatation can adequately compensate for the reduction in effective arterial pressure due to sheath pressures of that magnitude or less. The damaging effect of 40 mm. of pressure indicates that it would probably be futile to expect therapeutic benefit from pressures much above 30 mm. on the recovery of the mouse's tail from any type of insult.

The increase in necrosis found for the higher pressures seems to be a rough measure of the decrease in blood flow (ischemia) due to the inability of vasodilatation to compensate for the consequently greater reductions in effective arterial pressure. The sharp increase in necrosis observed between the 70 and 80 mm. pressures probably marks the reduction in average flow due to the onset of intermittent flow as the sheath pressure exceeded the diastolic pressure.

The average length of tail surviving 100 mm. of pressure (6.5 mm.) approximated a width of cuff (8 mm.) used in estimating the systolic pressure, which for other groups of mice was found to be about 120 to 130 mm. Hg. If, as seems reason-

TABLE 4. SYSTOLIC BLOOD PRESSURE

	ANIMAL									AV.
	1	2	3	4	5	6	7	8	9	
	Systolic blood pressure in mm. Hg									
16-mm. cuff										
1st day.....	95	100	90	88	95	90	105	95	95	95
4th day.....	95	95	95	104	92	90	95	95	90	95
8-mm. cuff										
9th day.....	120	120	120	125	120	120	115	115	120	119
12th day.....	115	135	125	120	118	120	135	115	120	123

	ANIMAL									AV.
	10	11	12	13	14	15	16	17		
	Systolic blood pressure in mm. Hg									
8-mm. cuff.....	132	129	127	132	125	120	125	132		128
	Mean blood pressure in mm. Hg									
Plenthysmographic method (3) <sup>1</sup> .....	99	130	102	116	103	119	102	117		111

<sup>1</sup> The authors wish to thank Dr. John R. McQuillan for measuring these pressures.

able, it is assumed that the systolic pressure averaged the same for this group and that the arterial pressure gradient within the cuff approximated that within an equal length of sheath, it follows that the systolic pressure in the arteries at the distal margin of the viable tissue must have exceeded the sheath pressure by about 20 to 30 mm. Hg. Since the tissue pressure must have closely approximated the sheath pressure, this probably is a rough measure of the amount by which the systolic pressure must exceed the tissue pressure in order to insure a blood flow adequate to prevent necrosis.

The removal of fluorescein both during and after the application of pressure corresponded with the viability of the tail better than did its penetration. In general it penetrated for variable distances beyond the proximal margin of the necrosis that later became evident.

The use of fluorescein to demonstrate blood flow in the cuff method for measuring blood pressure has the virtue of convenience. The method gives comparative rather

than absolute values. These are higher than those observed when a plethysmograph is used to detect blood flow beyond a cuff of the same width. This is probably based on the difficulty involved in keeping an unanesthetized mouse quiet for long enough to detect the slower volume changes expected as the cuff pressure approaches the true systolic pressure. Movement by the mouse does not interfere when fluorescein is used. The cuff pressure, therefore, can be maintained at a given level for as long as is necessary to detect the minimum flows expected as it approaches the true systolic level. It is likely that the method may detect the highest peaks of pressure within an interval if the systolic pressure is varying appreciably.

#### SUMMARY

Uniform pressure was applied over the length of the tail of the mouse by a pneumatic sheath. Thirty mm. Hg pressure was well tolerated for as long as a week. Higher pressures within the infrasystolic range caused progressively more necrosis of the distal end. The subsequent survival of the tail corresponded with the removal of fluorescein from the skin both during and after the application of pressure. The systolic pressure was estimated by a tail-cuff method in which fluorescein was used to demonstrate blood flow past the cuff.

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# EFFECT OF CARBON DIOXIDE ON INTESTINAL MOTILITY

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WHEN an animal is killed by a blow on the head and its abdomen opened immediately, the intestinal tract is frequently seen in a state of violent activity. This is presumably due to the acute asphyxia which develops upon sudden cessation of the circulation. It has been previously shown in mice (1) and in rats (2) that anoxic anoxia alone (i.e., without accumulation of carbon dioxide, as in asphyxia) depresses the motility of the small intestine. The intestine of the dog is much more resistant, but is also depressed if the anoxia is potentiated with cocaine (3). The present study was undertaken to determine whether the accumulation of carbon dioxide alone can be responsible for increased activity of the intestine.

## METHODS

Dogs and rats were used; the experiments were performed upon the animals in pairs: one experimental and one control. Each pair was chosen so that they were of approximately the same body weight. Essentially, the technique of Macht was used: the dogs were given 30 cc. of a mixture of 10 per cent charcoal in 10 per cent gum acacia solution by stomach tube, and 3 minutes later placed in a chamber through which was run, for the experimental animal a carbon dioxide-oxygen mixture, and for the control animal, pure oxygen. The 3 minutes in normal air were allowed in order to give a chance for some material to enter the duodenum, since immediate exposure to carbon dioxide frequently paralyzed the stomach.

The animals were removed from the chamber 30 minutes after the charcoal mixture was placed in the stomach and killed with ether, which stops intestinal peristalsis (4). The small intestine was removed *in toto*, slit open, and the distance the charcoal mixture had traversed was measured.

The same procedure was followed with the rats, with the following exceptions: 2 cc. of charcoal was administered, the animals were not placed in the chamber until 10 minutes after intubation, and they were killed after a total elapsed time of 40 minutes. The extra time outside the chamber was found to be necessary to insure that the stomach would discharge into the intestine in a reasonable proportion of the animals.

Two samples of the gas mixture in the chamber were analyzed for carbon dioxide content in the Hempel burette during the experimental run, and an occasional sample during a control run. In no case did the concentration of carbon dioxide exceed  $1\frac{1}{2}$

per cent during the control periods. During the experimental periods the following concentrations were attempted: for dogs, one group 7.5 per cent carbon dioxide, a second group 15 per cent; for rats, one group 10 per cent, a second group 15 per cent and a third 20 per cent. The average concentrations as shown by the determinations were close to the figures indicated by the flow meters. The ranges of actual concentrations for each group are shown in table 1. Some analyses for oxygen concentration during both control and experimental periods were performed. Due to the fact that the air was not completely removed from the chamber, the animals never breathed pure oxygen; its concentration ranged up to about 75 per cent.

There were 10 experimental animals in each group, with 10 controls for each, except the group of dogs at 15 per cent carbon dioxide, which comprised 15 animals in the experimental group and 15 controls.

TABLE 1. EFFECT OF CARBON DIOXIDE ON MOTILITY OF THE SMALL INTESTINE

CONC. CO <sub>2</sub> , AVERAGE, %	CM. TRAVELED, CONTROL	CM. TRAVELED, EXPERIMENTAL	DIFFERENCE, CM.	P <sup>1</sup>	ACTUAL CONC. OF CO <sub>2</sub> , RANGE, %
<i>Dogs</i>					
7½	177	114	63	.013	6.0-8.7
15	181	99	82	<.001	12.9-17.6
<i>Rats</i>					
10	75	72	3	>.20	7.4-12.8
15	74	57	17	.018	14.6-17.7
20	80	57	23	<.001	18.8-23.4

<sup>1</sup> According to Fisher.

## RESULTS

The results are summarized in table 1. There was a reduction in motility of the small intestine of dogs at a carbon dioxide concentration of 7.5 per cent significant at about the 1 per cent level (Fisher's *t* test), and a more marked reduction at a concentration of 15 per cent, significant at the 0.1 per cent level. The rats showed no change at 10 per cent CO<sub>2</sub>, while there was reduced motility at 15 and 20 per cent CO<sub>2</sub>, the latter highly significant.

## DISCUSSION

These findings are consistent with the well-known fact that a shift of the hydrogen ion concentration of the body tissues toward greater acidity has in general a depressing effect. But the aforementioned stimulation of intestinal activity by rapidly developing asphyxia is not explained. Perhaps the speed with which the condition develops, or the simultaneous presence of acute and severe anoxia in asphyxia, or both, produces the different result. However, Bisgard and Johnson (5) noted inhibition of the intestine of dogs following the inhalation of pure carbon dioxide, which certainly produces a condition somewhat resembling asphyxia. This

procedure is rather drastic. Schnohr (6) has also reported a cessation of motor activity of the intestine following increased CO<sub>2</sub> concentration in the rabbit. His observation was made through a cellophane window in the abdominal wall.

The finding that the small intestine of the rat is more resistant to high concentrations of carbon dioxide than that of the dog was rather unexpected, inasmuch as it has been previously shown that anoxia has a much more pronounced effect on intestinal motility of rats and mice than that of the dog (1, 2).

#### SUMMARY

Dogs and rats were exposed to increased carbon dioxide concentrations ranging from 7.5 to 20 per cent, and intestinal motility determined by measuring the length of intestine traversed by a charcoal-acacia mixture during a given time. Motility was depressed in the dog by a concentration of 7.5 per cent or more, and in the rat by a concentration of 15 per cent or more.

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# STIMULATING EFFECT OF SUGAR, FAT, AND MEAT MEALS ON DUODENAL SECRETION IN THE DOG

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THE humoral control of the secretory activity of Brunner's glands was first demonstrated by Florey and Harding (1). Using animals with extrinsically denervated, transplanted pouches of the upper duodenum, they noted an increase in the volume of secretion following the ingestion of a mixed meal. This finding was confirmed by Sonnenschein, Grossman and Ivy (2) who also made preliminary investigations with 'pure' foodstuffs to determine which ingredients of the mixed meal were the effective secretagogues. These workers considered their evidence insufficient to draw any conclusions, and therefore the purpose of this investigation was to elucidate the question of secretagogues.

Secretagogues may act either by being absorbed and carried by the blood to the responsive cells, or by causing the release of a hormone which stimulates the glands. In support of the hormonal mechanism, both Florey and Harding, and Sonnenschein and coworkers showed that extracts of intestinal mucosa stimulated Brunner gland secretion. The former group believed that secretin was the effective agent in these extracts, whereas the latter group concluded that some constituent other than secretin was responsible.

## METHODS

In this investigation two types of preparations were used on 6 mongrel dogs: in the pouch type, dogs A, B and C, the supra-papillary portion of the duodenum was brought through an abdominal stab wound as a pouch and sutured to the abdominal wall with the open end of the pouch exposed; in the flap type, dogs D, E and F, the tube of duodenum, instead of being fashioned into a pouch, was brought through a stab wound, cut lengthwise along the antimesenteric edge, and sutured to the abdominal wall in the form of a rectangular flap. In both types the vascular pedicle was kept intact, and the continuity of the gastrointestinal tract was restored by end to side gastro-duodenostomy.

The secretion was collected by means of a funnel which led to a graduated centrifuge tube, from which the volume was read directly. The funnel surrounded but did not touch the exposed duodenal mucosa. The dogs were fasted for at least 15 hours before each experiment, and basal secretion was collected for 1 hour preceding the administration of various stimulants. The test meals were as follows: 50 gm. of dextrose in 100 cc. of water, 50 cc. of cottonseed oil, and 50 gm. of lean beef; 50 to 100 cc. of saline was used as a control. Except for a few instances all

test meals seemed to be enjoyed by the dogs, and in the exceptions the liquids were fed without difficulty by squirting small volumes directly into the back of the mouth with a syringe.

### RESULTS AND DISCUSSION

The results of control tests in which the rate of spontaneous secretion was measured, are presented in table 1, which was constructed according to the following method. The average values for the control hour and for each of the two following hours were calculated for the experiments on each dog; then, disregarding the number of tests on each dog, an unweighted average of these values was taken to determine

TABLE 1. RATE OF SPONTANEOUS SECRETION<sup>1</sup>

DOG	SEX	TYPE OF PREPARATION	NO. OF TESTS	CONTROL HOUR	60-MIN. COLLECTION PERIODS	
					1st	2nd
				cc.	cc.	cc.
A	F	Pouch	4	5.80	6.08	4.54
B	M	Pouch	3	3.17	3.00	2.32
C	F	Pouch	1	.65	.40	.30
<i>Mean</i> .....				3.21	3.16	2.39
<i>Per cent control level</i> .....				100	98	74
D	F	Flap	2	6.78	5.95	5.80
E	F	Flap	3	1.08	1.67	1.90
F	M	Flap	3	3.65	2.68	3.75
<i>Mean</i> .....				3.84	3.43	3.82
<i>Per cent control level</i> .....				100	89	100

<sup>1</sup> The derivation of the figures and construction of the table are explained in the text.

the mean, which in turn was transformed so as to be expressed as percentage of the control level.

In figure 1 are expressed graphically the results of 22 tests in which the response of duodenal pouches to sugar, fat and meat was measured. The percentage values in this and subsequent graphs were derived in a manner similar to that described in table 1. These values represent the percentage of the control level for the two hours following the ingestion of the test meals. The maximal responses were 159 per cent to sugar, 170 per cent to fat and 136 per cent to meat. It is clear that all test meals stimulate the glands in this type of preparation to increase their secretion. Both concentrated glucose and fat are depressants of gastric acid secretion, so it is unlikely that the stimulatory action of these foods is secondary to stimulation of acid secretion by the stomach.

It was found that the flap preparation did not respond in a similar manner, as shown in figure 2. For duodenal flaps, the maximal responses were 114 per cent of

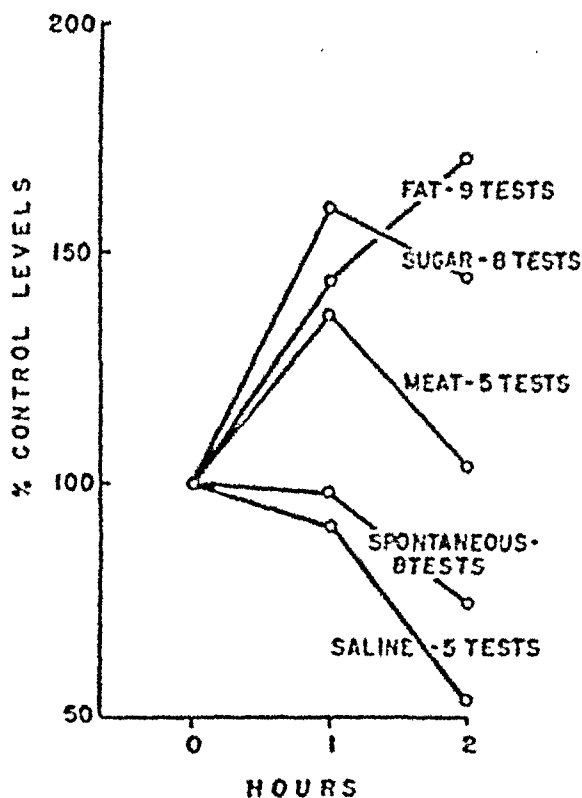


Fig. 1. RESPONSE OF POUCH TYPE preparation to various pure foodstuffs.

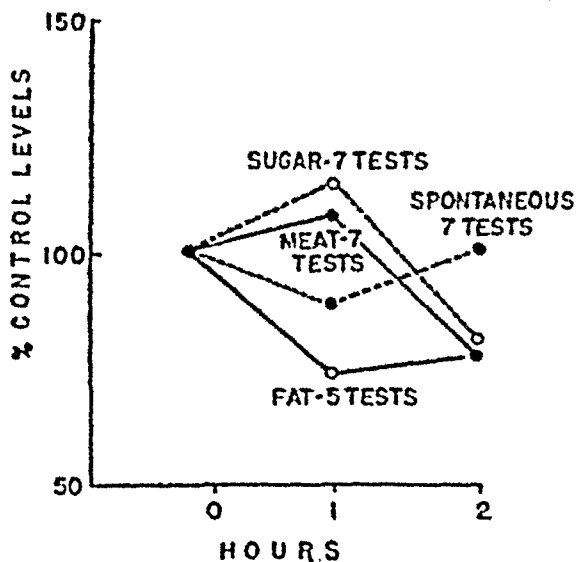


Fig. 2. RESPONSE OF FLAP TYPE preparation to various pure foodstuffs.

the control level to sugar, 78 per cent to fat, and 108 per cent to meat (50-100 gm.) These results indicate that the flap preparation is not significantly stimulated by sugar or meat, and may be inhibited by fat.

When a comparison is made of the secretory responses of pouches and flaps to a mixed meal (commercial dog food, Pard) the difference is clear (fig. 3). The volume of secretion from the pouch type increased to 182 per cent of the control level, while that from the flap type increased to only 120 per cent.

The difference in response to these two types of preparation might be attributed to the fact that mechanical stimulation due to rubbing together of the mucosal folds is eliminated in the flap type. That the flap type does have a secretory response is shown by the results of 6 tests in which 20 mg. of SI<sup>1</sup> was administered intravenously (fig. 4). SI is an intestinal extract containing secretin, cholecystokinin, pancreozymin and other unidentified substances. The injection of SI increased the rate of secretion from the flap to 182 per cent of the control level. Comparing this response with that of pouches to SI, where the rate of secretion increased to 165 per cent of

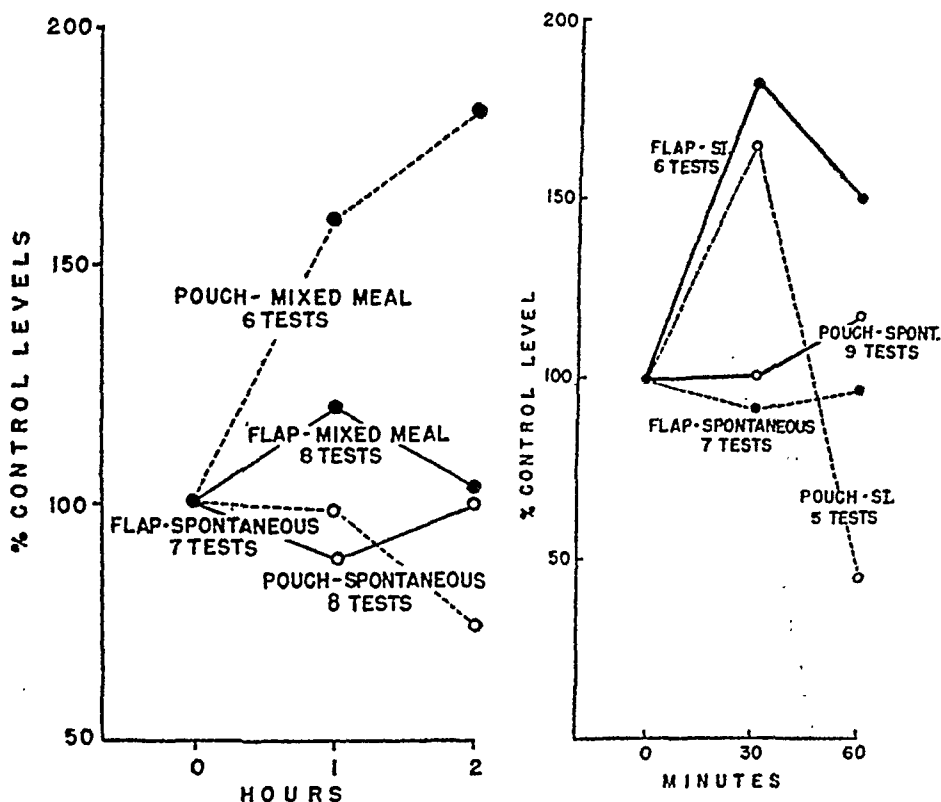


Fig. 3 (left). SHOWING THE DIFFERENCE between the flap and pouch type preparation in their responses to a mixed meal.

Fig. 4 (right). EFFECT OF SI (INTESTINAL EXTRACT) given intravenously in 20 mg. doses in the flap and pouch types of preparation.

the control level, we are led to conclude that the difference in response to a mixed meal is not due to a difference in responsiveness to this humoral agent.

Motility of the duodenum in anesthetized rabbits was also studied, using a balloon inserted into a 10-cm. length of isolated duodenum, and attached to a water manometer, which recorded contractions on a kymograph drum. The results of 5 experiments are shown in table 2. The number of contractions is seen to increase, on the average, from 6.2 to 9.6 per 5-minute period following the intravenous injection of 20 mg. of SI.

<sup>1</sup> The SI was kindly supplied by Dr. Leon Gershbein, University of Illinois.

The question arose concerning the degree to which motility can be held responsible for the differences observed between the responses of the pouch and flap.

TABLE 2. DUODENAL MOTILITY IN THE RABBIT FOLLOWING THE INTRAVENOUS INJECTION OF 20 MG. SI

TEST	NUMBER OF CONTRACTIONS PER FIVE-MINUTE PERIOD			
	Control	1	2	3
1	5	9	6	5
2	8	11	11	8
3	9	13	12	10
4	7	6	4	7
5	2	7	2	2
Av.....	6.2	9.6	7.0	6.4

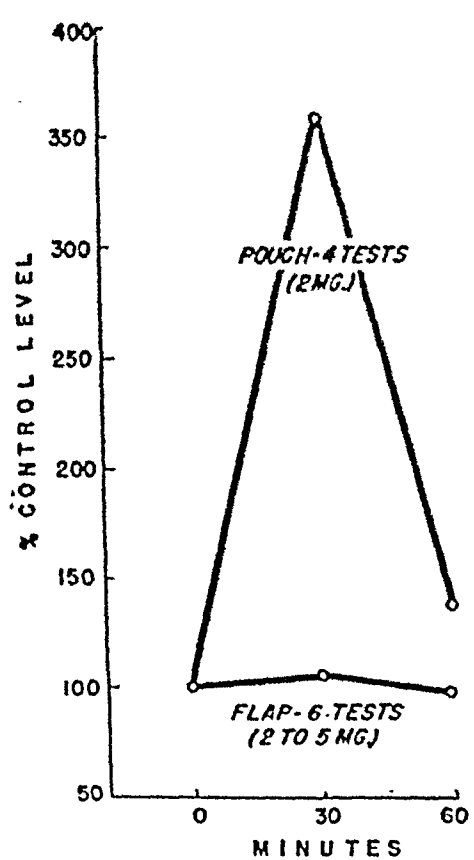


Fig. 5. EFFECT OF URECHOLINE on the secretion of Brunner's gland juice in dogs with flap and pouch types of preparation.

Important evidence on this question was gained when urecholine (2 mg. injected subcutaneously) was used as a stimulant for motility, and the pouch was found to respond markedly, 357 per cent of the control level, while the flap showed no response (fig. 5). Table 3 shows the 't' values for these and preceding tests.

Since the difference, then, in the maximal responses to SI is not great, and there is no large difference, therefore, in sensitivity, the difference in response to 'pure' and mixed meals may be ascribed to motility, causing rubbing together of the mucosal folds, and incidental mechanical stimulation in the pouch, which effect is largely absent in the flap type. This interpretation is supported by the results of a study by J. S. Ivy on the effect of pilocarpine on mucus secretion by the pyloric mucosa (3). He used animals with everted pyloric pouches, which prevented rubbing together of the mucosal folds, and found that in these pilocarpine did not stimulate the secretion of mucus.

Fogelson and Bachrach (4) showed that the secretory response of the duodenum to SI is accompanied by increased motility. These

findings, along with our study concerning SI-induced motility in the rabbit, and urecholine-induced motility in the dog, suggest the possibility that there may be 2 humoral factors: one stimulating secretion directly and the other stimulating motility which results in a secretory response secondary to mechanical stimulation.

If such a humoral stimulant for motility should be established by subsequent investigation, it would represent a difference between the first portion of the duodenum and the remainder of the small intestine, because careful studies of trans-

TABLE 3. STATISTICAL ANALYSIS OF THE MEANS OF THE TOTAL NUMBER OF TESTS ON EACH STIMULANT<sup>1</sup>

STIMULATING AGENT	POUCH				FLAP			
	No. of Tests	Control	1st Hour	2nd Hour	No. of Tests	Control	1st Hour	2nd Hour
		cc.	cc.	cc.		cc.	cc.	cc.
Sugar.....	8	3.88	6.13 $t = 3.7^{**}$	5.65 $t = 4.1^{**}$	7	2.94	3.43 $t = 1.0$	2.41 $t = 1.0$
Fat.....	9	3.18	5.23 $t = 3.1^*$	6.16 $t = 4.2^{**}$	5	3.46	2.57 $t = 2.0$	2.82 $t = 1.2$
Meat.....	5	2.93	4.17 $t = 1.6$	3.15 $t = 0.6$	7	2.92	3.16 $t = 1.0$	2.26 $t = 1.5$
Mixed meal.....	6	1.92	3.62 $t = 4.0^{**}$	4.03 $t = 8.4^{**}$	8	2.79	3.31 $t = 1.2$	2.81 $t = 0.07$
SI.....	5	Control 1.11	30 min. 1.76 $t = 5.0^{**}$	60 min. 0.50 $t = 2.9^*$	6	Control 1.44	30 min. 2.62 $t = 4.1^{**}$	60 min. 2.23 $t = 2.7^*$
Urecholine.....	4	2.30	8.25 $t = 4.5^{**}$	3.15 $t = 0.8$	6	0.69	0.71 $t = 0.01$	0.66 $t = 0.01$

<sup>1</sup> The 't' values were calculated for the differences between the control hour and each of the 2 following hours. One asterisk indicates that the value is significant at the 5% level, 2 asterisks, significant at the 1% level and no asterisk, that the probability is greater than 5%.

TABLE 4. SUMMARY OF THE EFFECTS OF VARIOUS STIMULANTS ON THE TWO TYPES OF PREPARATION

STIMULATING AGENT	RESPONSE	
	Pouch	Flap
Sugar.....	+++	0
Fat.....	+++	0
Meat.....	++ <sup>1</sup>	0
Mixed meal.....	+++	+ <sup>1</sup>
SI.....	++	+++
Urecholine.....	+++	0

+ = 20% increase over control level. ++ = 20% to 50% increase over control level  
+++ = over 50% increase over control level.

<sup>1</sup> Not statistically significant.

planted loops of small intestine have failed to reveal a humoral stimulant for motility in response to feeding (5).

The effects of the 'pure' foodstuffs used in this study must be determined in denervated pouch preparations before conclusions can be drawn concerning the

possible rôle of nervous mechanisms. Previous studies had of course established the existence of a humoral mechanism in the case of a mixed meal.

#### SUMMARY AND CONCLUSION

Table 4 presents a summary of results with various stimulating agents. Statistical analysis of the differences in secretory rates between the control hour and each of the 2 following hours, shows that all of the responses of the pouch are significant with the exception of the response to meat, and that the only significant response of the flap is that to SI (table 3).

It is therefore concluded that there is no single, specific secretagogue controlling Brunner's glands, and that the humoral agent may have 2 factors: one stimulating secretion directly, and the other stimulating motility.

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# SECRETION OF INTESTINAL JUICE DURING HYPERCALCEMIA<sup>1</sup>

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THE literature contains many conflicting reports on the influence of blood calcium concentration on the gastrointestinal tract. Hemorrhage, ulcers of the pyloric and duodenal mucosa, anorexia, diarrhea, vomiting and changes in the secretions of the stomach, as well as marked changes in the excitability of the nervous system, have been attributed to deviations from normal of the blood calcium. The earlier literature has been reviewed by Babkin, Komarov and Komarov (1). Schiffrin (2) reported that administration of parathyroid hormone to dogs with either a Pavlov pouch or a gastric fistula resulted in a diminished volume of gastric juice, and an increased concentration of pepsin but a decreased total output of enzyme. In the Heidenhain-pouch the volume of gastric juice was increased without affecting the concentration of pepsin, resulting in a greater total output of enzyme.

The investigation described below was undertaken in the hope of adding some information regarding the influence of hypercalcemia on the secretion of intestinal juice.

## METHODS

The experimental animals were two female dogs, maintained at constant weight (19 kg. each) on dog biscuits.<sup>3</sup> Except for a short period subsequent to the massive dosages of vitamin D, the animals were in excellent health. Loops of the jejunum were prepared and the intestinal juice collected as described by Nasset, Pierce and Murlin (3). Fasting experiments were begun 16 hours after the last meal; feeding experiments were begun 30 minutes after the ingestion of one-half the daily ration of dog biscuit.

Invertase was determined by incubating one ml. of the homogenized intestinal juice with 24 ml. of 12 per cent sucrose for 16 hours at 38°C. An aliquot was deproteinized with zinc sulfate and sodium hydroxide, the amount of reducing sugar present in the filtrate determined by the method of Somogyi (4), and the concentration of enzyme expressed as milligrams of reducing sugar produced. Peptidase was determined by incubating one ml. of homogenized intestinal juice with 24 ml. of 5 per cent peptone<sup>4</sup>, at pH 7.8, for 16 hours at 38°C. An aliquot was taken, and the amino nitrogen determined by the Sørensen formol titration as described by Dunn and Loshakoff (5). The concentration of peptidase was expressed as milligrams of amino nitrogen released. Total production of either

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<sup>2</sup> The data in this paper are taken from a thesis submitted by Harold R. Murdock, Jr. to the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree Master of Science, 1948.

<sup>3</sup> Purina Laboratory Chow.

<sup>4</sup> Difco-Bacto.

enzyme was expressed as the product of total volume of juice and activity per unit volume. Toluene served as the preservative during the incubation of the enzymes. All glassware used in the collection of the juice and enzyme incubation was boiled in strong soap solution and rinsed with glass distilled water. Control tests of both enzymes were made using boiled intestinal juice.

Blood calcium determinations were made according to the Tisdall method as modified by Clark and Collip (6). The intestinal juice was analyzed for calcium (7) and chloride (8). Carbon dioxide capacity was determined after equilibration of the sample with a mixture of 5 per cent carbon dioxide and 95 per cent oxygen at 38°C. (9). The pH was measured with a glass electrode.

It was desired to raise the blood calcium concentration by several different methods and for this purpose parathyroid extract, vitamin D, and calcium gluconate were used.

The parathyroid extract<sup>6</sup> was given subcutaneously on Sunday evening and in order to coincide with the maximal effect on blood calcium, which occurs in 12 to 18 hours (10), the first collection of juice was begun Monday morning, 12 hours after injection of the extract. Subsequent collections were made on Wednesday and Friday of the same week and another injection given the following Sunday. It was expected that this scheme might reveal immediate as well as delayed effects of the parathyroid extract. Dog 1 was given 5 injections ranging from 35 to 60 U.S.P. units. Dog 2 was given 2 injections, one of 95 units and the other of 380 units.

Vitamin D<sub>2</sub><sup>6</sup> was prepared by dissolving enough vitamin in sesame oil to make a solution that contained 1,000,000 U.S.P. units/ml. Dog 1 was fed 950,000 units (50,000 units/kg. body weight) daily with its food. Dog 2 was given the same daily dose, but the solution was placed in a gelatin capsule which was given orally. Dog 1 received 5 doses of 950,000 units each. Dog 2 received 5 doses of 950,000 and one of 350,000 units.

Calcium gluconate was obtained as a 10 per cent solution in 10 ml. ampoules.<sup>7</sup> Over a period of 10 minutes enough (13-14 ml.) of this solution was given intravenously to raise the blood calcium concentration to approximately 3.60 mm/l. (14.4 mg. %). In order to maintain this high level of blood calcium, a dilute solution of calcium gluconate (6 gm. in 250 ml. of isotonic saline) was given slowly by intravenous drip for the first 64 hours of each experiment.

Controls, in which 250 ml. of isotonic saline were given intravenously, were compared with the calcium gluconate experiments. The parathyroid extract injection and vitamin D experiments were compared with the normal by taking the average of the results obtained in control periods before and after each experimental period.

## RESULTS

The experimental results are summarized in tables 1 and 2.

*Parathyroid Extract.* The highest blood calcium concentration obtained with dog 1 after parathyroid extract injection was 2.75 mm/l. (11.0 mg. %) and with dog 2 3.25 mm/l. (13.0 mg. %). The parathyroid extract brought about, in most cases, a decline in both total volume of intestinal juice and enzyme production in the feeding as well as the fasting experiments. This was especially pronounced with dog 2. The standard errors, however, are so great that these changes cannot be considered statistically significant. In dog 2 there was a progressive decline in volume and enzyme production on succeeding days of the week after the parathyroid extract was given.

*Vitamin D.* The large doses of vitamin D brought about, in both dogs, a toxic condition, which was first noticed on the day after the fifth dose was given. Whenever the animals tried to eat, the food was vomited immediately and they remained in a lethargic condition for several days.

<sup>6</sup> Eli Lilly and Company.

<sup>6</sup> Crystalline Calciferol provided by Winthrop Chemical Co.

<sup>7</sup> Bristol Laboratories Inc.

TABLE 1. EFFECT OF HYPERCALCEMIA ON THE SECRETION OF INTESTINAL JUICE

Dog 1

PERIOD	NO. OF EX- PERS.	VOLUME	INVERTASE (INVERT SUGAR)	PEPTIDASE (AMINO NITROGEN)	BLOOD CALCIUM	
					Aver- age	Highest
<i>Fasted</i>						
		ml.	mg/7 hr.	mg/7 hr.	mM/l.	
Control 1.....	6	13.7 $\pm$ 1.1 <sup>1</sup>	4747 $\pm$ 722	226 $\pm$ 27	2.58	2.60
Parathyroid.....	7	11.2 $\pm$ 0.9	3263 $\pm$ 369	220 $\pm$ 20	2.59	2.75
Control 2.....	6	13.7 $\pm$ 1.5	3747 $\pm$ 581	270 $\pm$ 37	2.55	2.60
Vitamin D.....	8	12.4 $\pm$ 1.2	2565 $\pm$ 253	191 $\pm$ 19	3.26	3.72
Control 3.....	7	11.5 $\pm$ 2.1	4853 $\pm$ 826	250 $\pm$ 46	2.60	2.70
Saline control.....	4	27.0 $\pm$ 4.5	4308 $\pm$ 346	307 $\pm$ 36	2.50	2.50
Ca. gluconate.....	6	16.8 $\pm$ 1.9	5400 $\pm$ 512	321 $\pm$ 13	3.56	4.00
<i>Fed</i>						
Control 1.....	6	14.7 $\pm$ 1.9	4525 $\pm$ 1098	203 $\pm$ 34	2.45	2.51
Parathyroid.....	7	15.3 $\pm$ 1.7	3206 $\pm$ 230	270 $\pm$ 57	2.58	2.65
Control 2.....	4	16.0 $\pm$ 2.9	2644 $\pm$ 139	223 $\pm$ 58	2.49	2.49
Saline control.....	4	32.1 $\pm$ 3.8	3786 $\pm$ 190	302 $\pm$ 39	2.50	2.50
Ca. gluconate.....	6	14.8 $\pm$ 2.7	2689 $\pm$ 383	181 $\pm$ 21	3.55	3.75

$$^1 \text{Standard deviation of the mean} = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

TABLE 2. EFFECT OF HYPERCALCEMIA ON THE SECRETION OF INTESTINAL JUICE

Dog 2

PERIOD	NO. OF EX- PERS.	VOLUME	INVERTASE (INVERT SUGAR)	PEPTIDASE (AMINO NITROGEN)	BLOOD CALCIUM	
					Average	Highest
<i>Fasted</i>						
		ml.	mg/7 hr.	mg/7 hr.	mM/l.	
Control 1.....	8	25.7 $\pm$ 2.1 <sup>1</sup>	12650 $\pm$ 1615	433 $\pm$ 41	2.63	2.70
Parathyroid.....	5	21.7 $\pm$ 2.0	7469 $\pm$ 1426	321 $\pm$ 51	2.84	2.95
Control 2.....	6	19.3 $\pm$ 1.8	6874 $\pm$ 718	269 $\pm$ 25	2.60	2.60
Saline control.....	5	23.1 $\pm$ 1.2	4645 $\pm$ 966	223 $\pm$ 25	2.55	2.55
Ca. gluconate.....	4	18.4 $\pm$ 3.4	7755 $\pm$ 1018	272 $\pm$ 33	3.50	3.84
Control 3.....	8	30.6 $\pm$ 1.7	14879 $\pm$ 760	568 $\pm$ 20	2.44	2.45
Vitamin D.....	5	16.4 $\pm$ 1.1	8666 $\pm$ 552	394 $\pm$ 16	4.00	4.72
Control 4.....	5	40.6 $\pm$ 1.8	18953 $\pm$ 1970	708 $\pm$ 40	2.58	2.68
<i>Fed</i>						
Control 1.....	7	23.2 $\pm$ 1.0	8669 $\pm$ 1405	338 $\pm$ 42	2.65	2.70
Parathyroid.....	4	18.3 $\pm$ 2.6	3002 $\pm$ 484	228 $\pm$ 15	2.74	3.25
Control 2.....	5	28.0 $\pm$ 2.1	3108 $\pm$ 288	199 $\pm$ 21	2.60	2.62
Saline control.....	5	33.6 $\pm$ 4.6	4257 $\pm$ 852	261 $\pm$ 47	2.58	2.58
Ca. gluconate.....	4	33.2 $\pm$ 3.7	5411 $\pm$ 1312	286 $\pm$ 42	3.44	3.80

$$^1 \text{Standard deviation of the mean} = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

Vitamin D in *dog 1* induced a slight reduction in volume of intestinal juice as well as 40 per cent and 27 per cent decreases in total invertase and peptidase output respectively. The values, however, are not significantly different from the controls. *Dog 2* showed a significant diminution in both volume of intestinal juice and total enzyme output when the vitamin D was given. From figure 1 it can be seen that the changes became pronounced on the fourth day after the first dose of vitamin D, coinciding precisely with the peak concentration of blood calcium. The reduced secretion persisted for several days after withdrawal of vitamin D from the diet, but the experiments were discontinued because the dog consistently re-

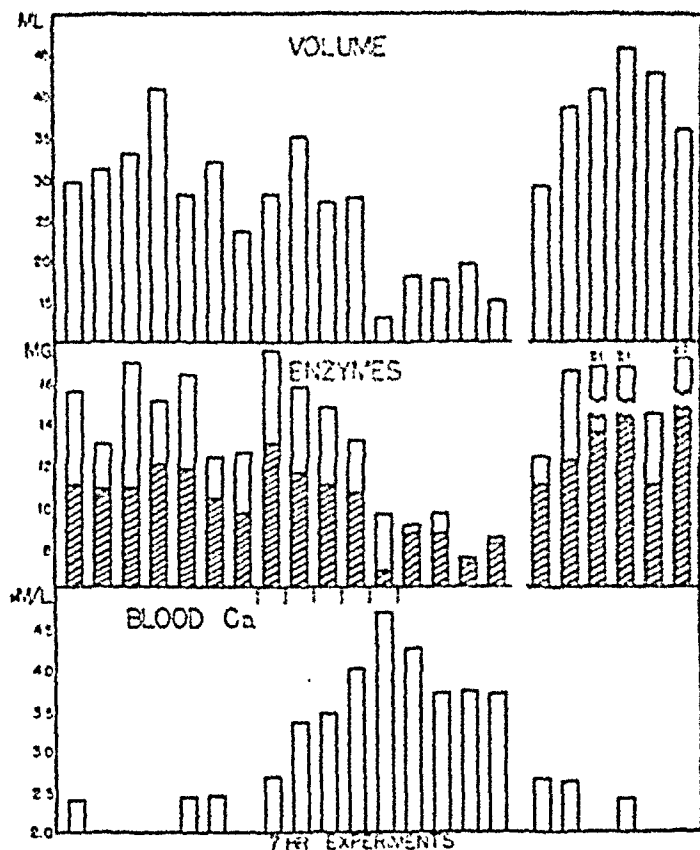


FIG. 1. EFFECT OF VITAMIN D (calciferol) feeding on intestinal secretion. *Dog 2*, calciferol fed as indicated by the arrows; the first 5 doses were 930,000 U.S.P. units each and the sixth 350,000 units. The break in the enzyme and volume base line indicates a recovery period of 11 days. Invertase (mg. reducing sugar  $\times 0.001$ ) is indicated by height of clear bar; peptidase (mg. amino N  $\times 0.01$ ) is indicated by hatched bar.

fused food. As soon as the dog regained its appetite and strength, control experiments were resumed and, as seen in figure 1, the blood calcium concentration, volume of intestinal juice, and enzyme output returned to normal.

The pH of the intestinal juice of *dog 2* during the vitamin D period ranged from 7.90 to 8.44. The pH during the control period ranged from 7.82 to 8.48. The chloride and carbon dioxide capacity were 146 mM/l. and 17.1 mM/l. respectively during the vitamin D period and 145 mM/l. and 17.5 mM/l. respectively during the control periods.

**Calcium Gluconate.** The calcium gluconate caused a significant diminution in the volume of intestinal juice during the fasting experiments of both dogs and the feeding experiments only with *dog 1*. The total invertase and peptidase output was increased in all instances except the feeding experiments with *dog 1*, but

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## THE MEDULLARY ORIGIN OF RESPIRATORY PERIODICITY IN THE DOG<sup>1</sup>

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THE currently dominant view of the nature of respiratory periodicity envisages the interplay of two factors (1-3). The first is presumed to be a basic inspiratory tetanus without periodicity representing the unmodified, inherent activity of the medullary inspiratory center. It is then postulated that periodic inhibition of this discharge by impulses of extramedullary origin segments the basic tonic inspiratory rhythm into alternating periods of inspiration and expiration. Impulses arriving via the Hering-Breuer reflex afferents in the vagi are supposed to be one of two factors which can inhibit periodically the fundamental inspiratory tetanus and permit expiration. In addition, the upper reaches of the pons are considered to contain an ill-defined area connected with the medullary respiratory center in such a way that a reverberating suppressor circuit is generated that can in the absence of the vagi periodically inhibit the fundamental tonic inspiratory discharge of the medullary center. This circuit implied the existence of fiber-tract connections between the inspiratory center and the pontine center by which pontine activity is initiated by the inspiratory discharge. A return pathway from the pons to the expiratory center is inferred to mediate expiration and inhibit inspiration (1).

Arrest of inspiration, whether by the Hering-Breuer reflex or the ponto-medullary suppressor circuit, is thus considered to be affected by processes which are set in play by the inspiratory act itself; in the absence of the vagi and the pons, therefore, inspiration ought to be continuous and uninterrupted. This is apparently what does occur; in fact, the whole structure of the hypothesis outlined above rests solely upon

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the well-verified observation that animals with transection of the neuraxis below the upper portion of the pons pass into a state of inspiratory spasm or 'apneusis' when the vagi are sectioned or disabled. The whole concept of the mechanism of respiratory periodicity thus revolves, for the present, around the interpretation placed upon the phenomenon of apneusis; the theory as outlined above rests upon the view that apneusis is a 'release' phenomenon uncovering a submerged tendency of the medullary center through deprivation of regulating influences.

This hypothesis runs counter to the classical view endowing the medullary respiratory center with an inherent capacity for generation of the periodic discharges mediating the respiratory act. Neurophysiology affords examples of such local origin of complex phasic acts, such as the locomotory alternation of flexion and extension carried out by a completely deafferented portion of the spinal cord isolated by transection. To date, however, experiments purporting to reveal phasic respiratory activity in isolated segments of the medulla have lacked conviction because of inclusion of the pons (1).

Objections to the hypothesis of medullary aperiodicity have been expressed (4, 5); these have centered in part around the obvious difficulties of transection procedures: hemorrhage, shock and anoxia are difficult to combat, especially in the dog, and have been recognized by all who have attempted this type of experimentation. The preliminary report of Nicholson and Hong (6) is of the greatest importance, but has never been fully detailed. To date, the proponents of the theory of respiratory periodicity of medullary origin have failed to find a satisfactory alternative explanation for apneusis, which has remained an impregnable bulwark of the opposing theory.

The experiments reported here are presented because they appear to provide such a satisfactory explanation for the phenomenon of apneusis. They suggest that apneusis is not a primary phenomenon inevitably following low decerebration and vagotomy, but a secondary occurrence which can be produced or abolished at will, and demonstrated to be not the inherent output of the uninhibited inspiratory center, but a superimposed drive, 'occluding,' in the Sherringtonian sense, the normal output of the medullary center. It arises in the main, as shall be described, from an exaggerated inspiratory responsiveness characteristic of animals with pontine decerebration.

#### METHODS

Forty-one dogs were employed, all procedures being carried out under ether or pentothal anesthesia until decerebration. Since adequate control of hemorrhage by carotid ligation and vertebral compression is almost never achieved in the dog, as it can be in the cat, the basilar artery was exposed on the floor of the brain stem by rongeur anteriorly from the foramen magnum, and occluded by ligature or silver clip at the level of the pons or trapezoid body, in a modification of the procedure of Pollock and Davis (7). The base of the skull was approached by a parapharyngeal route which avoided the cranial nerves. The carotid arteries were either ligated or left free, and the carotid bodies left intact or denervated by section of their nerve supply and novocainization of the free stumps according to the demands of the individual experiment. While in early experiments the vagi were disabled reversibly by cooling, in later experiments they were sectioned, often early in the procedures. Artificial respiration was required for variable intervals in some experiments. Simple thoracic compression or insufflation with air was adequate in most instances. Gelfoam and thrombin were employed to

reduce hemorrhage in some experiments. Adequate time for recovery from the anesthesia was allowed after the first mid-collicular transection. Subsequent transections and vagotomy were carried out after respiration was stabilized. Records were obtained by means of an accordion-type pneumograph. Figure 13 shows the levels at which transections were made and summarizes the results.

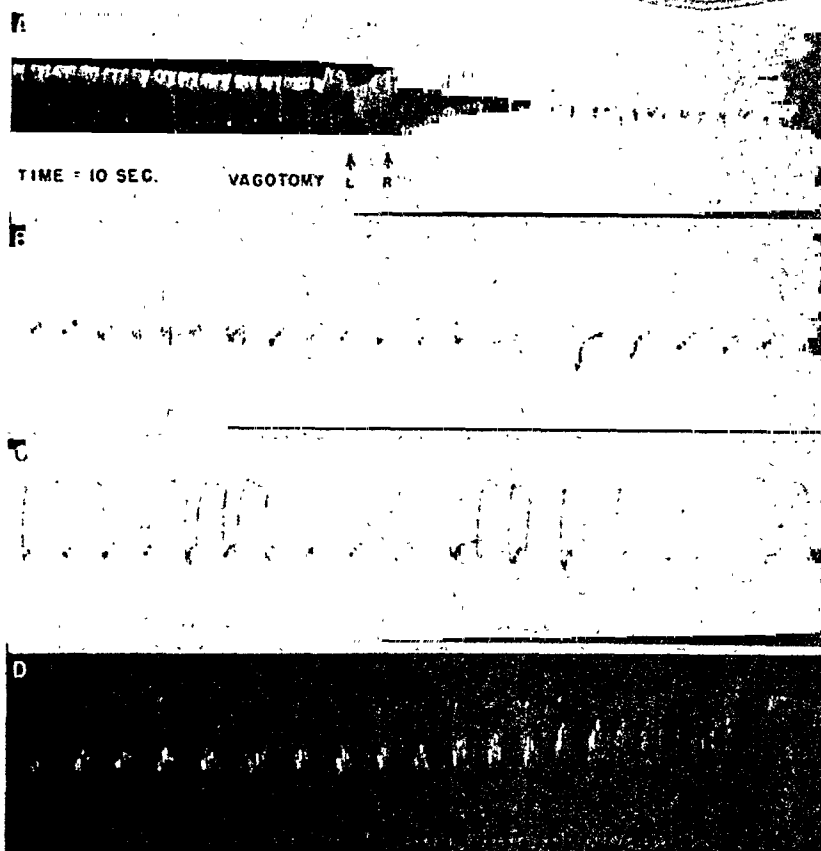


Fig. 1. DOG B 23. Jan. 21, 1949. 16 lb. female. Carotids ligated and basilar artery occluded at lower border of pons. Carotid innervation intact. Mid-collicular section followed by upper pontine section. Survival 1 hr. 15 min. after vagotomy. In all figures except 4 and 13, inspiration is downward. A. Control, pontine section before vagotomy, rapid breathing with regularly spaced deep respirations. Appearance of apneustic breathing after vagotomy. Phasic respiratory movements can be seen at the height of each inspiratory spasm. B, C, and D. Continuous records until death. In D the phasic movements increase in amplitude as inspiratory spasm lessens, and the last respirations are of normal character.

## RESULTS

### *Apneusis in the Dog*

*Apneusis is not permanent.* While the apneusis that develops after vagotomy in the pontine decerebrate cat is reported to be pronounced and prolonged—long enough in experiments reported by most investigators to cause asphyxial death—it is by no means as prolonged in similar preparations in the dog. After a variable period, which may last as long as a minute or more in some cases, the inspiratory spasm is broken by an expiration. In a few seconds inspiratory spasm reappears, and the cycle is repeated, producing a picture, not of apneusis, or continuous inspiratory spasm, but of apneustic breathing, in which almost equal phases of inspiration

and expiration alternate, usually at rates of one or two per minute (figs. 1-3, 6, 7, 9, 10). This modified respiration maintains oxygenation of the blood sufficiently to maintain life, and such preparations have lived for hours. It is in fact the wide fluctuation in blood pressure accompanying the violent respiratory alternations which is the main cause of death by producing excessive hemorrhage around the brain stem.

In the dog, then, vagotomy in the classical pontine decerebrate preparation does not unmask a permanent inspiratory state, but induces a condition in which, although inspiration is intensified and prolonged, periodic expiration does occur, and a respiratory rhythm is maintained.

*Apneusis is not total.* In the cat, it is reported, apneusis appears on vagotomy in the pontine decerebrate preparation as a state of inspiratory spasm totally replacing

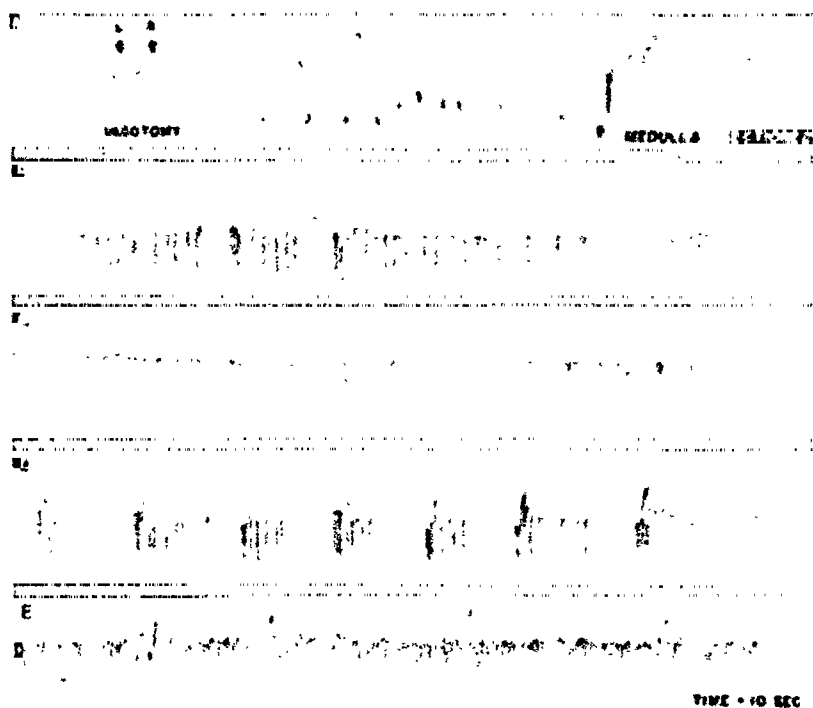


Fig. 2. DOG B 25. Jan. 25, 1940. 26 lb. female. Carotids ligated, sinuses intact. Pontine section. vagi intact. A. Breathing with double periodicity characteristic of pontine decerebration with vagi intact. Apneustic breathing produced by vagotomy. Further medullary section with restoration of normal breathing. B, C, D, and E. Sample strips at intervals until animal was killed 6 hrs. 14 min. after medullary section. Biot's breathing in D was abolished by removal of clots from cerebellar space.

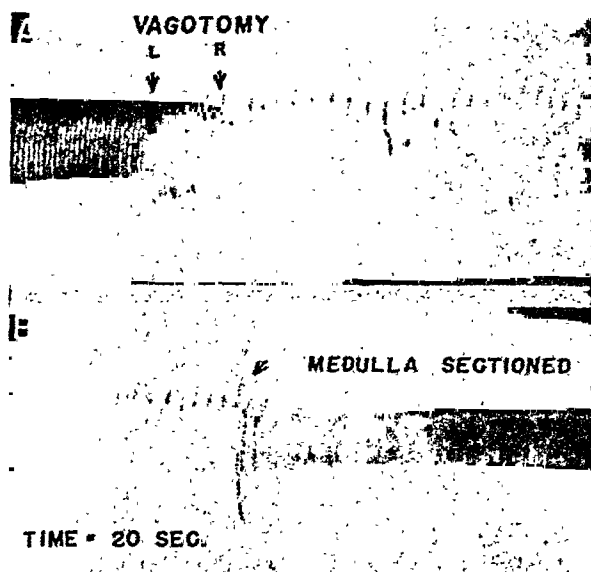
the respiratory alternation it supplanted. In the dog this may occasionally be the case, but in most experiments there remain some vestiges of periodic respiration superimposed, as it were, on the phases of apneustic breathing. When apneustic breathing is most complete, thorax and abdomen may show no signs of normal periodic respiration, but it can be noted, especially in the moments before the inspiratory phase, that the accessory respiratory muscles of the face are contracting periodically as though the animal were breathing normally. In most preparations however the abdominal and thoracic muscles show shallow respiratory waves superimposed upon

the inspiratory spasms of apneustic breathing (figs. 1, 4-7, 9, 10). These may be so small as to be unregistrable with the conventional accordion-type pneumograph, but often they are of sufficient magnitude to maintain significant pulmonary ventilation (cf. figs. 6, 9). Abdominal breathing contributes relatively more to these movements than thoracic, although the thorax does participate.

Whatever their value in ventilation may be, the presence of such periodic respiratory movements demonstrates the existence of a respiratory periodicity at fairly normal rates even at the height of apneusis or inspiratory spasm. Even during apneusis, therefore, some part of the respiratory complex must remain periodic.

*Apneusis disappears in the deteriorating preparation: normal respiration reappears.* In all preparations in which apneustic breathing was permitted to persist until death, it disappeared as the condition of the preparation deteriorated. The depth and dura-

Fig. 3. DOG B 14. Dec. 10, 1948. Mid-collicular decerebration with vagi cut. In A, an incomplete pontine section produced apneustic breathing which was abolished with restoration of normal breathing by an incomplete medullary transection in B. Animal killed in good condition 1 hr. later.



tion of the apneustic intervals diminished, the extent of the superimposed phasic respirations increased in equal proportion, and apparently normal respiration reappeared for some minutes before death (fig. 1). The last respiratory acts of the apneustic animal were thus essentially normal respirations. If deterioration and death be considered to be attended by progressive collapse of the more sensitive centers, and the survival of fewer and fewer of the more resistant structures and functions, then the reappearance of normal breathing must be regarded as the emergence of a simple act from a more complex one, not, as the theory of Pitts, Magoun, and Ranson would imply, the reestablishment of a complex act out of a simpler one.

The essential implication of the above three sections is clear, that apneusis represents some process superimposed upon a basic periodic medullary respiratory rhythm.

*Apneusis lessens and disappears upon further transection in the medulla.* When the brain stem of an animal showing apneustic breathing is again transected in the upper reaches of the medulla, apneustic breathing lessens and disappears (figs. 2, 3). Inspirations are no longer prolonged, expirations follow at the normal time, and each respira-

tory act resembles the normal. The pattern of respiratory periodicity may vary, as will be described later, but a true periodicity remains, without trace of inspiratory spasm. When the transection is made below the trapezoid body before vagotomy, vagotomy then fails to influence respiration (fig. 8A).

In some experiments, where pontine decerebration caused a long period of apnea for some reason or another, vagotomy also failed to produce apneusis (fig. 8C). Presumably anoxic damage to the anterior portion of the brain stem, either because of the apnea itself or circulatory changes that may have caused it, served as a physiological transection at the medullary level.

Apneusis, or apneustic breathing, or inspiratory spasm, is thus characteristic of a supramedullary preparation, and in no way is it an attribute of a medullary preparation. The medulla when separated from the pons is thus incapable of prolonged inspiratory spasm, but is normally periodic.

*Apneusis can be produced or enhanced by carotid body stimulation.* Consideration of the influences to which the brain stem remnant is exposed after pontine transection and vagotomy, in search of a peripheral factor that might contribute to the over-

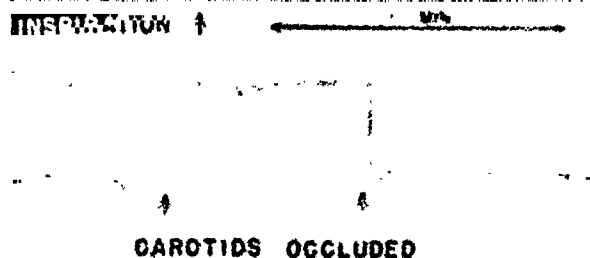


FIG. 4. 1896 at 3. Nov. 28, 1914. 20 lb. male. Pontine decerebration, vagi suppressed by repeated cooling. Carotids free. Carotids occluded, producing apneusis with superimposed abdominal respirations at 32/min.

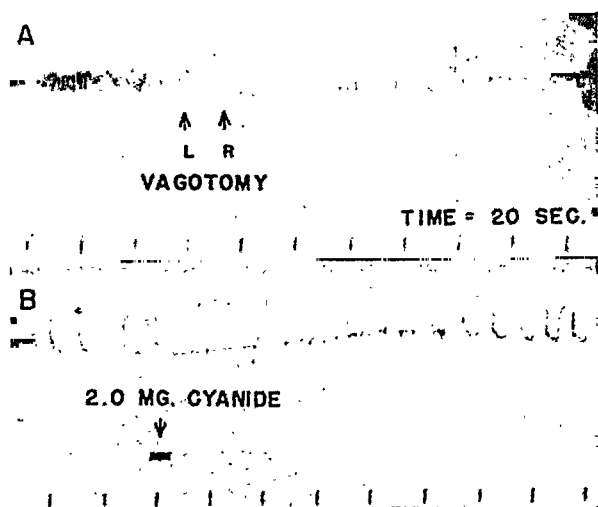
riding inspiratory drive that results in apneusis and apneustic breathing, led ultimately to the realization of the role of the carotid bodies. Bilateral ligation of the common carotids is a standard procedure in decerebration of the cat. Singularly enough, few authors who have written on the subject of apneusis have considered the state of the carotids; it is implied in Lumsden's account that at least one carotid was ligated for insertion of an arterial cannula for registration of blood pressure (8, 9). Stella has reported that intracarotid injection of cyanide can produce apneusis (10, 11). In all the experiments recounted above, in which vagotomy produced apneustic breathing in the pontine decerebrate preparation, the common carotids were ligated to reduce hemorrhage during decerebration. In other animals in which the carotids were later liberated, apneustic breathing also continued, and in these, apneusis could be prolonged by carotid ligation (fig. 4), or by intravenous or intracarotid injection of small quantities (1-6 mg.) of cyanide (fig. 5). Attempts were made to stimulate the sinus nerves electrically and on some occasions mild degrees of inspiratory spasm or prolongation of the inspiratory stage of apneustic breathing were obtained. In general, however, the effects of nerve stimulation were not so clear-cut as those with stimulation of the carotid bodies with cyanide.

*Apneusis is reduced by peripheral denervation of the carotid bodies or abolished by denervation of the medulla.* After apneustic breathing had been established by lower

pontine decerebration and bilateral vagotomy, subsequent denervation of the carotid bodies greatly reduced the duration and depth of the inspiratory periods, and augmented the underlying phasic component (fig. 6). In one experiment the procedure led to practically complete restoration of phasic breathing (fig. 7). In 4 experiments in which denervation was carried out as a preliminary procedure approximately 1 hour before decerebration, transection of the brain stem between the pons and trapezoid body followed by vagotomy failed to produce apneusis or apneustic breathing (fig. 8B), while the same procedure in animals with intact carotid innervation invariably produced apneusis and apneustic breathing.

Denervation of the brain stem stump in animals showing apneusis restored normal breathing (figs. 9, 10). In this procedure a probe was passed laterally between the medulla and the skull, severing, as autopsy later revealed, the eighth, ninth, and tenth cranial nerve roots, and the medullary components of the eleventh.

FIG. 5. DOG B 4. Nov. 2, 1948. 24 lb. female. Basilar artery tied, carotids looped during decerebration but thereafter free. A. Control, pontine decerebration before vagotomy. Production of apneusis for approximately 1 min. by vagotomy. Rhythmic abdominal respirations at 19/min. continue during apneusis. Apneustic breathing thereafter. B. Intravenous injection of 2.0 mg. potassium cyanide. Apneusis for 2 min. followed by continuance of apneustic breathing at 4 times/min.



It can therefore be concluded that apneusis, or apneustic breathing, or inspiratory spasm, represents an exaggerated inspiratory response superimposed upon, and practically completely occluding, the natural periodicity of the medullary respiratory center.

### *Respiration in the Medullary Preparation*

Whether or not the foregoing analysis of apneusis is correct in all its details, one salient fact emerges: that when the medulla is more completely denervated, either by section of the auditory and glossopharyngeal nerves or denervation of the carotid bodies, or by a medullary transection, all traces of apneusis disappear, and a fundamental respiratory periodicity re-emerges.

The respiratory activity of the medullary preparation is often indistinguishable from the breathing of the mid-collicular or the pontine decerebrate animal before the vagi are cut; there thus are no grounds for supposing that the respiration of the medullary preparation represents some more primitive or uncomplicated type of respiration different in its fundamental nature from normal breathing. In general,

however, it shows variations that suggest the absence of controlling influences, and three main patterns emerge: *a*) Regular, slow, and complete inspirations and expirations, suggesting an all-or-nothing discharge with each inspiration, are frequently seen (fig. 8*B*). Rates are not significantly different from normal, and neither polypnea nor extremely slow respiration are observed. *b*) Respirations of lesser amplitude which vary in depth and interval are the most common type (figs. 2, 9, 10). All gradations have been seen from completely regular spacing and depth to marked irregularity. This kind of respiration suggests a type of 'denervation ataxia' of a respiratory center deprived of its regulating influences, but it should be pointed out that pontine and mid-collicular preparations show some degree—and often as marked a degree—of

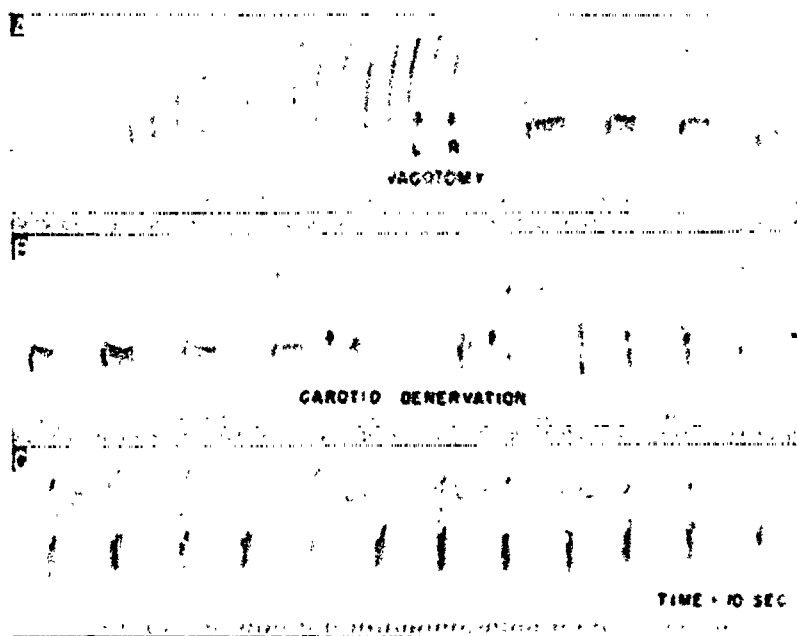


Fig. 6. DOG n 32. Feb. 7, 1949. 14 lb. female. *A*. Control. Pontine decerebration. Carotids intact and ligated. Vagotomy produces apneustic breathing without completely suppressing phasic respiratory movements. *B*, *C*. Denervation of carotids greatly diminishes the duration of inspiratory phases and increases the amplitude of the periodic respiratory movements.

this same irregularity when the vagi are still intact (figs. 7, 8*A*). *c*) Biot's breathing. This type of respiration appears as a transition between apneustic breathing and medullary respiration. The basic slow periodicity of the apneustic cycles remains, but the periods formerly devoted to inspiratory spasm are given over to more or less complete inspirations and expirations. Thus periods of regular phasic expirations and inspirations alternate with periods of apnea at the rate of apneustic breathing (fig. 11). Often some element of inspiratory spasm is associated with the periods of respiration and in fact almost all gradations between apneustic breathing and Biot's breathing may be found. In general, the impression was gained that Biot's breathing appeared when there was pressure on the brain stem or when apneusis was not completely suppressed by denervation procedures in high medullary preparations. Biot's breathing was not seen after low medullary transections, either because of the level of section or because in these experiments the cerebellum was completely removed to facilitate

transection and opportunity was therefore not afforded for development of pressure through accumulation of blood between the medulla and cerebellum. It was our impression that the level of section was a more important factor than hemorrhage or pressure.

#### SUMMARY OF RESULTS

*Levels producing apneusis or apneustic breathing.* In 20 experiments apneusis was produced at one time or another by vagotomy after low decerebration. It invariably appeared after sections between levels A (anterior) 1-P (posterior) 1 and A2-P<sub>3</sub> (fig.

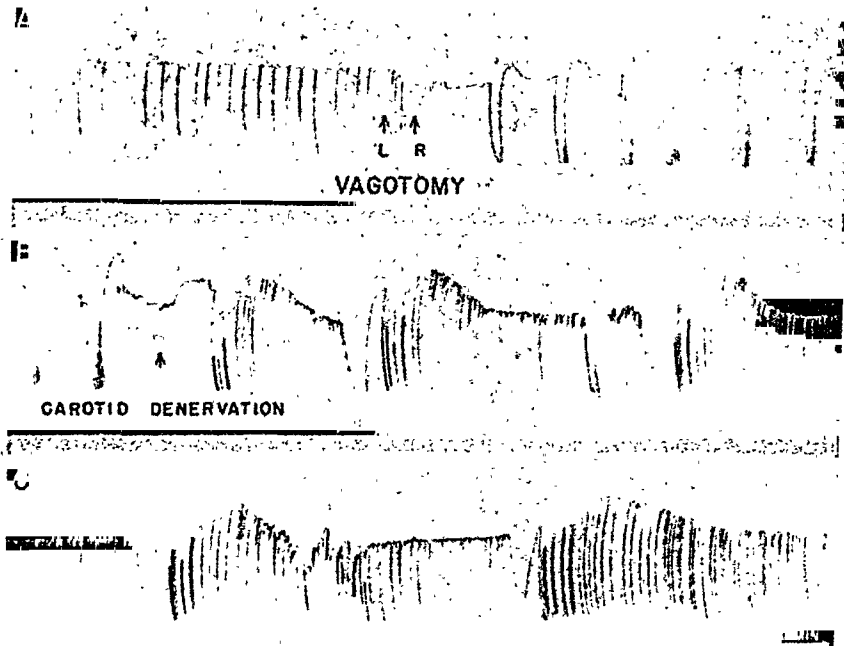


Fig. 7. DOG B 24. Jan. 24, 1949. 14 lb. male. Carotids ligated, basilar artery occluded at lower border of pons. A. Control record after pontine decerebration and with vagi intact. Note irregularity in depth and rhythm. Vagi sectioned with appearance of apneustic breathing which is incomplete. B. Denervation of carotid bodies and sinuses with disappearance of apneustic breathing. Resultant respiration is more rapid than control, is still irregular in depth and rhythm. Occasional periods of inspiratory spasm.

12), except in 2 experiments where a long period of apnea (approximately  $\frac{1}{2}$  hour) occurred after section at the level A2-P<sub>3</sub>. In these 2 instances it was presumed that anoxia had disabled the 'apneustic center.' The longest survival of an animal in apneustic breathing was 2 hours, 46 minutes, after which the animal was killed.

*Role of carotid denervation.* Apneusis or apneustic breathing was prolonged or intensified by carotid occlusion in 4 experiments, and in 4 other experiments cyanide had the same effect. In 7 animals with low decerebration at levels anterior to A2-P<sub>3</sub>, in which the carotid bodies were carefully denervated before decerebration, apneusis or apneustic breathing failed to occur after vagotomy. The carotids were denervated after apneusis was produced in 4 experiments with reduction or disappearance of apneusis in 3 and no effect in the fourth. The medulla was denervated intracranially

in 3 experiments with complete restoration of normal breathing in all 3 instances. Survivals were 56 minutes, 1 hour 15 minutes, and 5 hours 30 minutes after medullary denervation.

*Normal respiration after medullary section.* The medulla was sectioned at levels A4-P5 or A4-P4 in 10 animals and in none did apneusis appear. In the 2 animals mentioned above normal respiration persisted after sections at the level A2-P3. Survivals ranged from 35 minutes to 6 hours 14 minutes after the final medullary section had been made. In none of these experiments was artificial respiration used after medullary section and vagotomy.

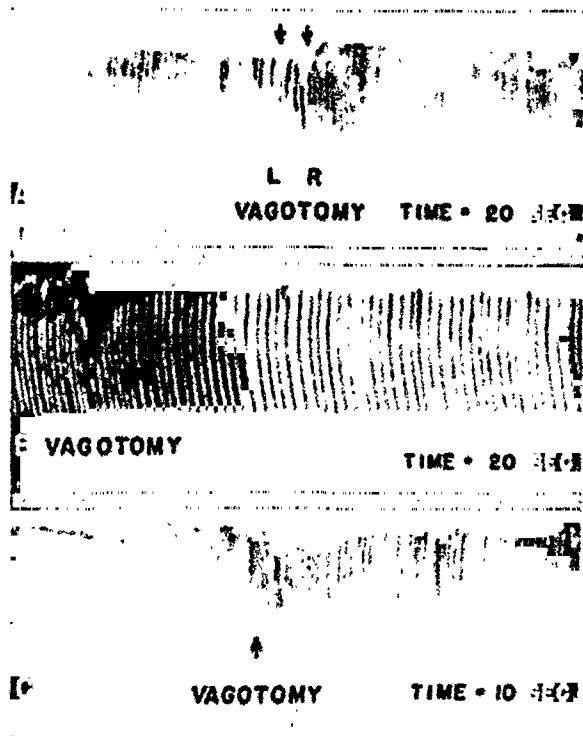


Fig. 8. A. DOG B 7. Nov. 22, 1948. 23 lb. female. Carotids not ligated. Section below trapezoid body. Vagotomy without essential change in respiratory pattern. Killed in good condition 1 hr. thereafter. B. DOG B 11. Dec. 1, 1948. 19 lb. female. Carotid bifurcation denervated. Section between medulla and trapezoid body. Vagotomy without effect. Survival for 35 min. thereafter. C. DOG B 28. Jan. 28, 1949. 17 lb. female. Mid-pontine section followed by  $\frac{1}{2}$  hr. apnea. Restoration of spontaneous respiration. Section of vagi without influence. Survival for 1 hr. thereafter.

### *Polypneic Panting in the Decerebrate Dog*

Despite reports of the existence of 'polypneic panting' in the decerebrate dog, this type of breathing was never encountered. This may be attributed in part to the absence of excessive hemorrhage and shock in these experiments and to the fact that in most instances the carotids were either denervated or were occluded only temporarily or not at all. This latter possibility is supported by some observations pointing to enhanced responsiveness to intravenous injection of cyanide or carotid artery occlusion below the bifurcation in the decerebrate dog. Rates above 60 per minute were not encountered even following cyanide injection or carotid occlusion (fig. 13).

### *Autonomic Imbalance*

A striking difference in autonomic balance was noted between the mid-collicular and pontine decerebrate preparations, the latter being characterized by a marked

bradycardia and hypersecretion of saliva. A detailed account of this phenomenon will be given elsewhere.

#### DISCUSSION

The most significant result of these experiments is the fact that a dog deprived of both pons and vagi can continue to breathe for long periods, 5 to 6 hours in many

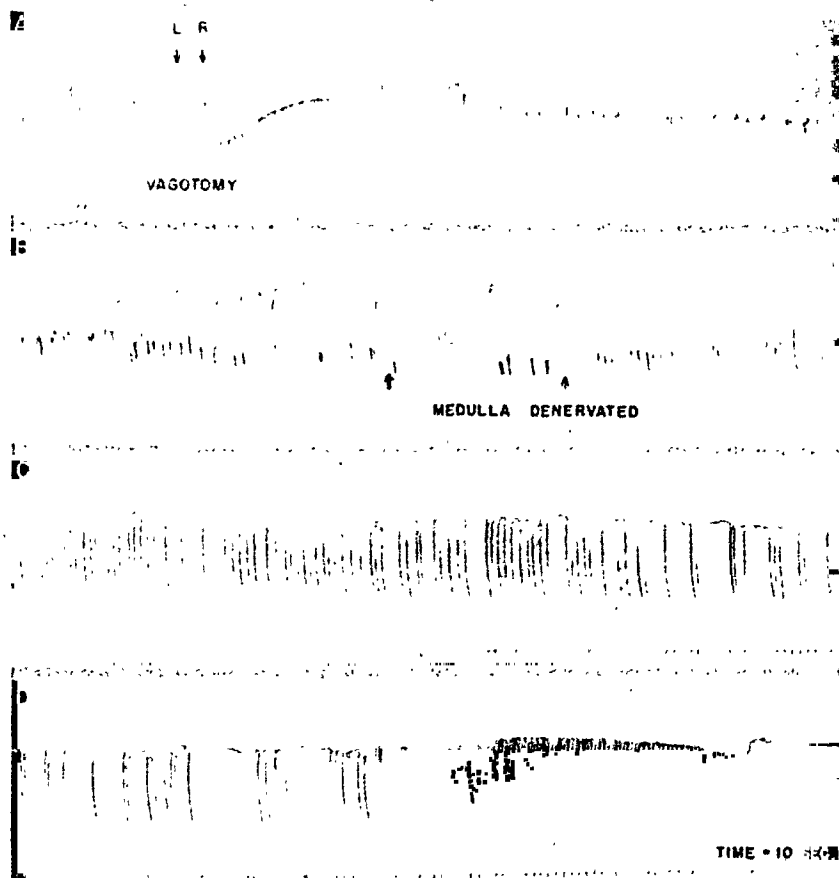


Fig. 9. DOG B 27. Jan. 27, 1949. 27 lb. female. *A*. Control. Pontine decerebration. Carotids intact and ligated. Section of vagi produces apneusis lasting 5 min., with relatively large phasic respirations superimposed. Thereafter apneustic breathing for 13 min. *B*, *C* and *D*. Records continuous with *A*. In *B* the sides of the medulla were stripped with return of normal respiration, which is at first more rapid and more regular than the control record before vagotomy. In this figure as in fig. 1, the deep respirations at a slow rate may have some relation to the slow rate of the subsequent apneustic breaths.

instances, in a fashion that cannot be distinguished in its essentials from that of the classical mid-collicular preparation, with or without the vagi.

Apneusis, or apneustic breathing, has been demonstrated to result from a superimposed phenomenon, appearing only at certain intermediate levels of transection, enhancing inspiration and altering the fundamental periodicity of the medulla without, however, completely extinguishing it in the dog. This exaggerated inspiratory drive results from the interplay of peripheral and central factors: the peripheral factor appears to be in the main the anoxic stimulation of the carotid bodies; the central

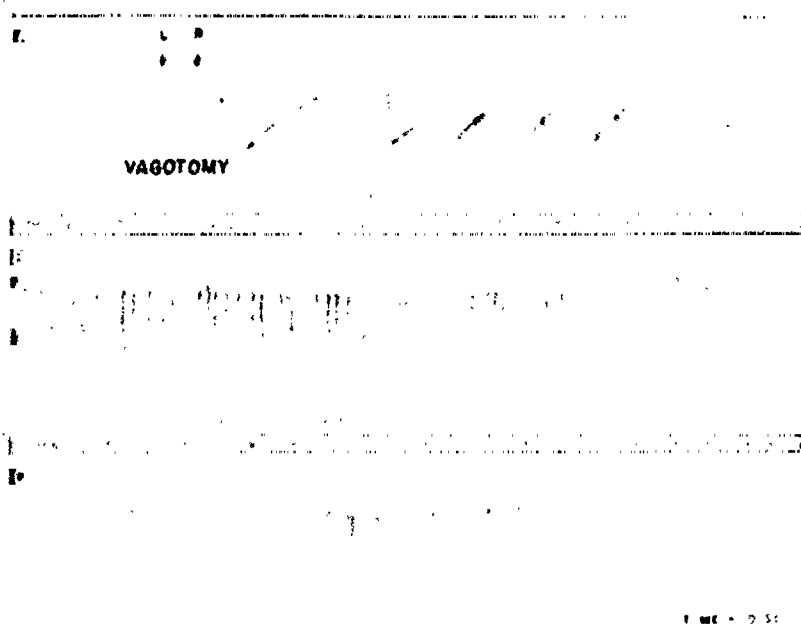


Fig. 10. DOG B 30. Feb. 1, 1949. 13 lb. female. Pontine decerebration. Vagi and sinuses intact. Carotids ligated. *A.* Vagi cut with production of apneusis for 3 min. (base line changes are the result of a leak in the recording system). Thereafter apneustic breathing. Noticeable respiratory oscillations in apneusis and apneustic breathing. *B.* Medulla stripped with restoration of normal breathing. *C.* Further section lower in medulla without alteration of breathing. Total survival after restoration of normal breathing, 1 hr. 15 min.

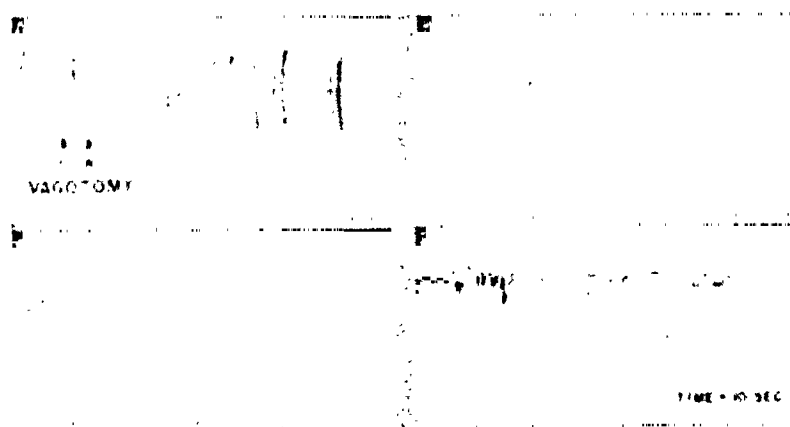


Fig. 11. DOG B 20. Jan. 14, 1949. Carotids denervated and ligated. High medullary transection. *A.* Biot's breathing, unaffected by vagotomy. Periods introduced by increase in respiratory tonus. *B.* Breathing still periodic but at longer periods. *C.* Periodicity lost but respiration irregular. Some postural changes remain. *D.* Essentially normal respiration. Total survival after vagotomy 1 hr. 30 min.

factor is the overfacilitation of inspiration which occurs when the lower part of the pons is included with the medulla in the intact portion of the brain stem.

The central effect on respiration of anoxic stimulation of the carotid bodies thus appears to be largely inspiratory in its influence, and it is no doubt expressed in the intact animal both in the increment in phasic respiration and in the increased in-

spiratory tonus of the anoxic animal, which Greene and Swanson (12) and Harris (13) have noted.

The facilitation of inspiration which occurs in the apneustic animal may well represent the activity of a portion of the facilitatory center to which Rhines and Magoun (14) have called attention, the anterior portions of which appear to facilitate phasic movements while the posterior portions facilitate tonic mechanisms. It would not be entirely coincidental, according to this view, that much the same levels of

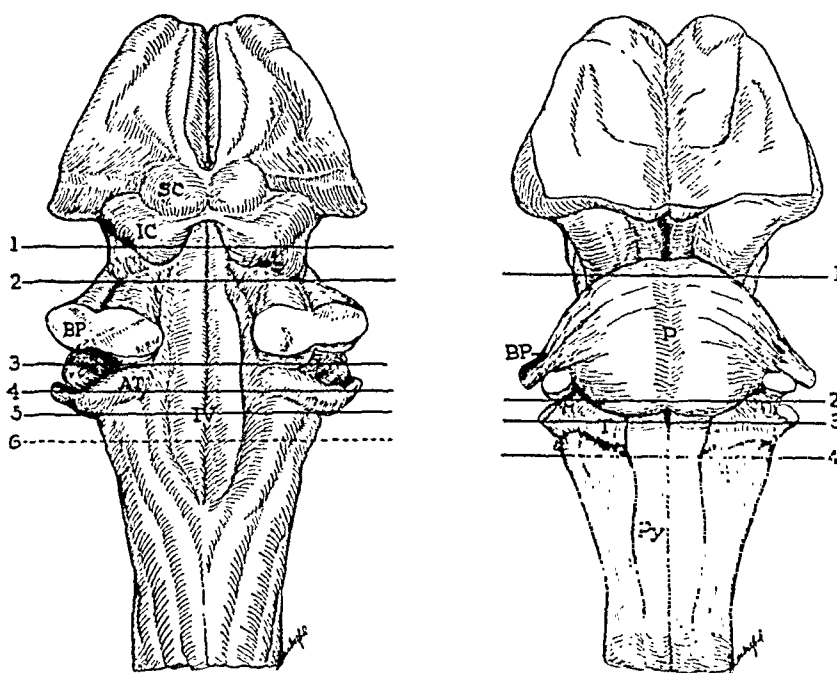


Fig. 12. DRAWING OF THE BRAIN STEM of the dog showing the levels at which transections were made in these experiments. S. C. superior colliculus; I. C. inferior colliculus; B. P. brachium pontis; A. T. acoustic tubercle; IV fourth ventricle; P. pons; T. trapezoid body; Py. pyramid. Transections at or below lines 1 or 2 posteriorly and line 1 anteriorly regularly produced apneusis or apneustic breathing after vagotomy. This persisted after transections as low as line 3 posteriorly and line 2 anteriorly. When transections were made at the general levels of line 4 posteriorly and line 3 anteriorly, apneusis persisted in some form although transitional types were commonly seen. With sections below line 5 posteriorly and line 4 anteriorly, apneusis was not seen and normal respirations alone occurred. The lowest transection made in these experiments was that reported in *Dog B 25* (see figure 2) in which the section passed between line 6 posteriorly and slightly behind line 4 anteriorly.

section produce the exaggerated facilitation of postural extensor reflexes which results in decerebrate rigidity on the one hand and apneusis on the other. It is of course clear that the two functions do not overlap completely, and inspiratory and extensor facilitation are not mediated by precisely the same segments of the facilitatory center, but there seems to be a large degree of correctness in the views of Henderson and Sweet (15), Teregulow (16), and others, who have regarded apneusis and apneustic breathing as related to decerebrate rigidity. If, finally, the hyperactivity of extensor reflexes in the decerebrate animal is considered to result from a denervation paralysis of a medullary inhibitory center (17), with a resultant imbalance in favor of facilitation, the midbrain centers which achieve this in the case of inspiration must be located in

part more posteriorly than those related to extensor reflexes, for while a mid-pontine transection in general only augments decerebrate rigidity, it is essential to the production of apneusis. The so-called pneumotaxic center can thus be considered as a part of the general neuronal masses which discharge into the medullary inhibitory center. This may well account for the lack of precise localization of the so-called pneumotaxic center.

The view of the nervous regulation of respiration which these experiments suggest is thus:

*a)* The medullary respiratory center is inherently periodic.

*b)* Respiration, like other basic mechanisms of the cord and bulb, is under the influence of modifying factors from external sources.

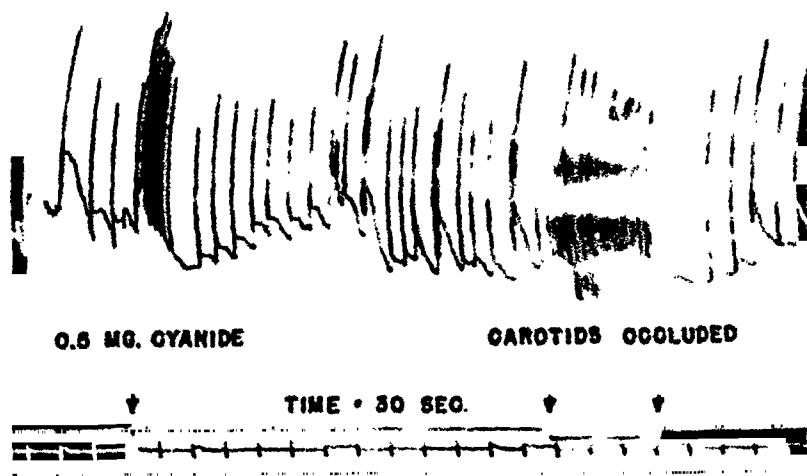


Fig. 13. DOB M O. Feb. 20, 1945. 15 lb. male. Mid-collicular section with carotids free. Intravenous injection of cyanide (0.5 mg.) or carotid occlusion increases respiratory rate to 40 and 50/min. respectively. Inspiration upward.

*c)* The posterior reaches of the midbrain facilitatory system enhance inspiration; this is normally kept in check by 1) the Hering-Breuer reflex and 2) the midbrain centers which activate the bulbar inhibitory regions. This mechanism is no different from that regulating other reflex phenomena in the cord, except for recognizable differences in anatomical representation in these centers, which, longitudinally speaking, are fairly extensive.

*d)* When by mid-pontine section inspiration is excessively facilitated, and impulses creating inspiratory drive impinge upon the system, apneusis is produced in the absence of the inhibitory effect of the Hering-Breuer reflex. In the dog this rarely occludes all traces of a basic respiratory periodicity even at the height of the inspiratory spasm.

*e)* Apneusis so produced can be abolished by appropriate section of the cranial nerves, or by section of the medulla at levels below the facilitatory center and/or the intramedullary tracts of the cranial nerves. While the glossopharyngeal nerve appears

to be of paramount importance, it was considered possible that the vestibular nerve was also involved, as it is in the maintenance of decerebrate rigidity in the cat.

The whole foundation of the concept "that the rhythm of breathing is impressed upon the respiratory center by inhibitory mechanisms operating from without; it is not an expression of properties inherent in neurons of the center," (1) rests solely upon the interpretation of apneusis as expressing the basic capacity of the denervated inspiratory center; in fact the phenomenon of apneusis is the only evidence for the ponto-medullary circuit theory of respiratory periodicity. Apneusis, as a phenomenon, is so invariably obtainable with certain procedures that often no further support for the hypothesis has been considered to be required. In the present experiments, however, apneusis in the dog has been demonstrated to be neither permanent nor total, even when most highly developed. Moreover, it has always been possible to eliminate it completely by one or several procedures and to restore normal breathing. Apneusis has been considered to be the activity of the fully denervated medullary center. Quite to the contrary it appears to be the result of an incomplete simplification of the respiratory system; on further simplification normal breathing has reappeared. Apneusis thus loses the primary significance which has been attributed to it in accounting for the respiratory rhythm.

In this light the dying return to normal breathing exhibited by all animals showing apneustic breathing is worth recalling. According to the ponto-medullary circuit theory the approach of death must stimulate the development of a previously silent center and pathways somewhere in the preparation which can again periodically inhibit the fundamentally tetanic discharge of the medulla. According to the same concept, many animals and humans ought to show apneustic breathing at death. It is more in accord with the concept that dissolution and approaching death reduce instead of increase the complexity of neuronal acts and interneuronal organization (18) to suppose that apneusis is a factor superimposed upon a basic medullary periodicity which falls away as less persistent centers cease to function, leaving finally the primary activity of the more resistant and more caudal respiratory center.

#### SUMMARY

Apneusis in the dog is neither permanent nor total nor an invariable consequence of low decerebration and vagotomy. The denervated medullary preparation is capable of normal periodic respiration. Apneusis represents a phenomenon superimposed upon the basic medullary activity. The medullary respiratory center is inherently periodic.

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# LOCALIZATION OF THE SITE OF ACTION OF A PULMONARY IRRITANT, DIPHOSGENE

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**K**NOWLEDGE of the locus of action of a toxic agent is basic to understanding its mechanism of action. In 1942, when an effective therapy or prophylaxis was being sought for phosgene poisoning, the site of action of the agent was still uncertain. Laqueur and Magnus (1) had argued from the rapid hydrolysis rate (2, 3) that inhaled phosgene would be destroyed before it could be carried away in the blood, and therefore must act only in the lung. Most workers (4-9) agreed and concluded that changes elsewhere were secondary to developing pulmonary edema, anoxia, dyspnoea, etc.; but some pathologists (10-14) insisted on an extra-pulmonic action of phosgene on the brain, liver, heart and kidneys.

The present experiments were designed to localize the site of action of inhaled phosgene. They were of the following sorts:

*Concerning Localization to the Lung.* 1) Gassing one lung only; 2) Gassing one of a cross-circulating pair of dogs, and attempts to find a circulating toxin; 3) Parapulmonic administration of the toxic agent.

*Concerning Localization Within the Lung.* 4) Attempts to differentiate between bronchiolar and alveolar effects; 5) Comparison of ordinary and massive doses of the agent.

## METHODS

For unilateral exposures a technique was devised for occluding one bronchus which proved similar to that already described by Jacobaeus for broncho-respirometry (15). Dogs were lightly anesthetized with nembutal (which had been shown not to alter phosgene pathology (16), or, in control experiments, mortality) and laid supine with a block under the neck to keep the trachea straight. With the mouth held open and the neck extended it was easy, under direct bronchoscopic visualization, to slip a small (1-3 cc. vol.), collapsed, condom rubber balloon, tied over the open end of a fine metal tube (1-2 mm. i.d.), into either major bronchus. The balloon was then inflated and the bronchoscope either removed or left in place to assure a clear airway. An alternative, but less satisfactory method was to place a Foley catheter in a bronchus, inflate the cuff, and then either close off the catheter to protect the lung or gas the catheterized lung alone through the catheter. Either lung may be permanently plugged to prevent edema fluid overflow by placing a soft rubber stopper in a bronchus with a Jackson forceps. Such a plug is usually not gas tight.

For crossed circulation experiments, carotid to jugular anastomoses were made under nembutal, heparin or chlorazol fast pink injected, and blood pressure followed in the carotid artery. Blood distribution and pressure were regulated by clamps on the connecting tubes.

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<sup>1</sup> This work was done during 1942-44 under a contract recommended by the Committee on the Treatment of Gas Casualties between the Committee on Medical Research and the University of Chicago.

Diphosgene was used throughout for convenience of handling. The findings are, however, almost certainly equally valid for phosgene, since, so far as tested, they act alike (16a).

### RESULTS

*Effects of Gassing One Lung Only.* The results of several types of unilateral exposure experiments are shown in table 1, and the appearance of unilaterally gassed lungs in figure 1. In 23 of 28 experiments (83%), the protected lobes were structurally normal, there being no alveolar or peribronchiolar edema, emphysema or bronchioli-

TABLE 1. EFFECT OF UNILATERAL GASSING IN DOGS (DIPHOSGENE)

EXPOSURE, mg/l. $\times$ min.	NO. DOGS	EXPERIMENTAL PROCEDURE	MORTALITY			PATHOLOGY
			Died		Lived	
			No.	Hr. sur- vival, aver- age	No.	
0.75 $\times$ 30	7	Lower right bronchus plugged permanently (rubber stopper)	7	12	0	Right lower lobes normal in 5; poorly fitted plug failed to protect in 3
	5	Lower right lobes alone gassed through catheter	4	6	1	Right lower lobes alone injured in 4. Both lungs damaged in 1
	1	Lower right lobes alone gassed—permanent plug then put into right lower bronchus			1	All lobes normal except right lower
0.71 $\times$ 30	6	Right lower lobes protected during exposure—permanent plug, then put into left bronchus	3	20	3	Right lower lobes normal in 5. Poorly fitted plug in 1
0.73 $\times$ 30	6	Right lower lobes protected during exposure. Postural drainage later	6	19	0	Right lower lobes normal in 5. Poorly fitted plug in 1
1.8 $\times$ 10	3	Right lower lobes protected during exposure. Postural drainage later	3	26	0	Right lower lobes normal in 3

tis. Unprotected lobes showed the typical changes (9, 17, 18) seen after ordinary exposure. In the 5 cases in which the 'protected' lung was damaged, it was established certainly or probably that the plugging was incomplete.

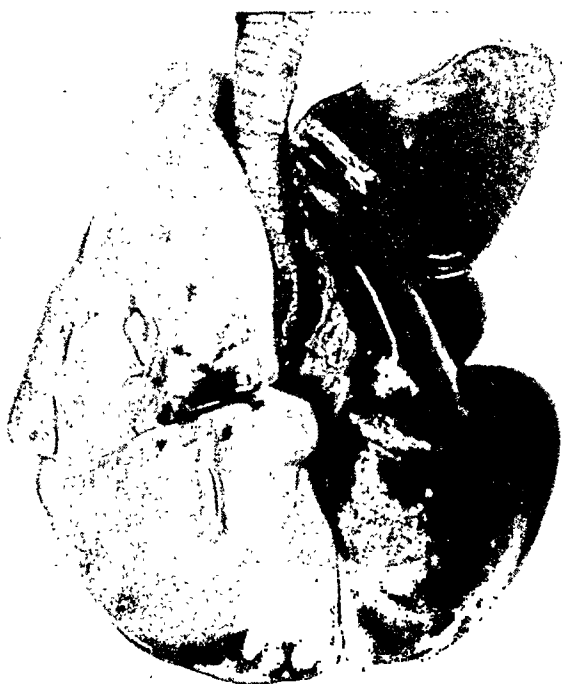
A number of animals with protected lobes died. In 7 the plug was left permanently in the protected side, preventing function of the normal lung. Nine were subjected to continual postural drainage and tracheal aspiration (16), which manipulation may have hastened death. Five animals gassed through a catheter were left with the gassed lobes unplugged and edema fluid from them flooded the normal lung and airways. Seven dogs were gassed with one lung plugged, following which the protected lung was freed and the gassed side plugged to restrain edema fluid. One of these ani-

imals died because of a poorly fitted plug. The remaining 4 survived far beyond the expected time of death and were killed at 42, 44, 77 and 93 hours after gassing.

Diphosgene, therefore, produces demonstrable pathological changes in the lung only after direct contact. A lung protected from such contact remains normal even though its mate be excessively gassed.

*Crossed Circulation Experiments.* In 2 experiments, one dog of the pair was gassed before crossed circulation began and the circulation was then continued for 5 and 1 hours, respectively. In each case the gassed dog died with typically progressing anoxaemia and pulmonary edema. One of the ungassed, cross-circulated dogs died, but with no pulmonary change or anoxaemia, and the other lived until killed 3.5 days later, apparently normal. In another 4 experiments, diphosgene gas was instilled

Fig. 1. UNILATERALLY GASED DOG LUNGS. Ungassed lung well aerated and expanded. Gassed lung solidly hepatized, edematous and practically air-free.



into the tracheal cannula of one dog while crossed circulation was in progress. All the poisoned animals died with typical edema. Three of the cross-circulated, ungassed animals died, but not until 11 to 38 hours later and with no pulmonary edema or other demonstrated change; one of them, killed 3 days later, showed no pathological change.

These two types of experiment not only indicate that diphosgene acts only and directly on exposed lung, but also that no toxic agent is released in significant amounts. Both blood and edema fluid from excessively gassed animals reached protected lungs, with no effect on them except for mechanical obstruction by fluid (17). Likewise, no systemic effects except those due to fluid loss appeared. More direct evidence of a toxic substance is also negative or ambiguous.

Liberation of histamine from a gassed lung has been reported (19, 20); but inhaled, atomized histaminase does not lessen the post-gassing syndrome nor delay death, and intravenous histamine does not aggravate the syndrome or accelerate

death (16). Large amounts of edema fluid from heavily gassed goats, suddenly injected intravenously into normal goats may kill,<sup>2</sup> but such deaths are due to multiple embolism (17). Intravenous injection of normal lung extract, being rich in thromboplastin, can similarly produce emboli and death. The edema fluid inconstantly contains a capillary permeabilizing factor. Small amounts given intradermally on the rabbit abdomen cause local leakage of previously injected Evans Blue. The effect is a feeble one, not comparable to that caused by vesicular fluid from mustard or lewi-

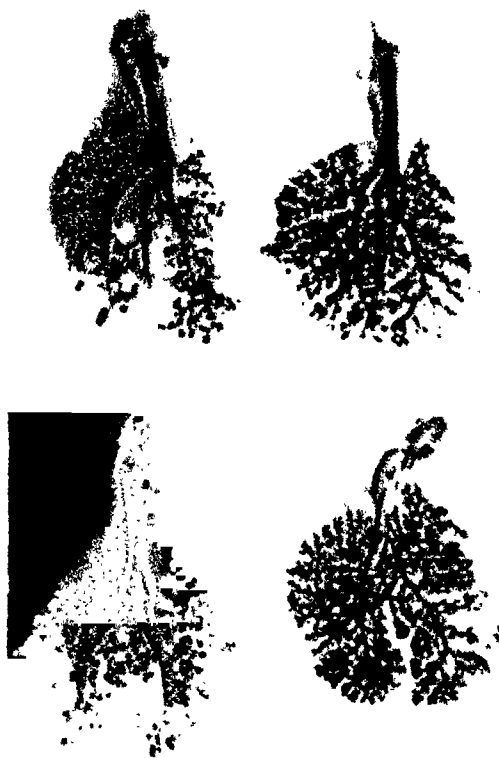


Fig. 2. NORMAL AND GASSED RAT LUNGS after administering  $\text{BaSO}_4$  suspension intratracheally.

site lesions. The factor of dilution in the large volume of edema fluid must, however, be noted. Finally, the transfusion of large amounts of blood from heavily gassed dogs to normal ones has no demonstrable deleterious effects (16).

*Parapulmonic Administration of Toxic Agent.* Recently obtained data (21, 22) show that the LD-50 for phosgene in dogs is about 1 mg/kg. by inhalation. For the purpose of these experiments, done before such data were available, we estimated the amount retained by a dog during a normal exposure to be about 1 to 2 mg/kg. This corresponds to 0.5 to 1 mg/kg. for diphosgene liquid, which is much easier to give parapulmonically than is phosgene gas. Four dogs were, therefore, given 1 to 2 mg. of diphosgene per kg. intravenously in 50 per cent alcohol in about 1 minute. None showed ill effects and, when killed 1 to 4 days later, autopsy revealed no pathological change.

<sup>2</sup> Done in collaboration with Captain R. W. Dougherty, V. C., of Dugway Proving Ground.

Given intraperitoneally, 85 mg. of diphosgene in 5 cc. alcohol produced discomfort, abdominal rigidity, ascites and death in 6 hours. At autopsy the peritoneum was necrotized, as it was also after the injection of a few drops of pure diphosgene liquid. Such large quantities undoubtedly hydrolyze to yield sufficient HCl (peritoneal surface was acid to litmus) to denature protein. There was, however, no pulmonary or other non-local effect. A smaller dose, 18 mg., injected into the abdomen in mineral oil had no deleterious effect, nor did as much as 5 cc. of the saturated vapor given intraperitoneally in rats.

*Attempts to Differentiate Between Bronchiolar and Alveolar Effects.* Bronchioles and bronchi are unquestionably damaged. Post-gassing bronchitis and bronchiolitis have been seen almost constantly by us and others (1, 9, 17, 18). Emphysema also occurs early after exposure (16, 18, 23), and can be tentatively ascribed to prompt and transient bronchial narrowing. Trypan blue, injected into mice before gassing, appears in the perivascular peribronchiolar edema fluid before it is visible in alveolar edema fluid (17), and the same sequence in appearance of edema is seen with standard staining techniques (18). Dogs (23) recovered from phosgene poisoning or goats after repeated exposures (22) may show fibroblastic proliferation originating in the bronchi and bronchioles, which also attests earlier injury to this mucosa.

Any bronchial narrowing is important, whether active or passive, transient or enduring. Histologically, bronchoconstriction has been only infrequently demonstrated (18). Dyspnoea, occurring shortly after exposure, can sometimes be relieved by vagotomy, various sympathicomimetic spasmolytics, or atropine (16, 24, 25, 26); but a comparable reduction in tidal air is not so relieved (1). Our own studies on anesthetized intact dogs, given short concentrated exposures by tracheal intubation, show a drop in tidal air which can be reversed by vagotomy (table 2). This effect of vagotomy could result from dilatation of uninjured rather than constricted tubes, or even from altered respiratory rate and excursion. Certainly vagotomy has little influence on the changes in intrathoracic pressure.

Shortly after exposure, intrathoracic pressure measurements show increased negativity on inspiration and increased positivity on expiration (table 3). The mean is displaced toward the positive side, which supports the clinical impression of a greater interference with expiration than with inspiration. Such a valve-like effect, perhaps involving bronchial narrowing, would also explain the development of emphysema.

Also in accord with the notion of bronchial narrowing is our finding that rat lungs passively collapse far more extensively before than after gassing. Thus, rat lung volumes have been measured by water displacement in a graduated cylinder. The average residual air in the passively collapsed normal lung is 0.4 cc., in the gassed lung (30 min. or more after the start of exposure), 1.7 cc.

We have failed, on the other hand, clearly to demonstrate constriction by 3 direct methods: 1) The rate of pleural transudation of fluid from trachea through pleura (27) was not altered by diphosgene in the highest doses (table 4). This is in contrast to reports of prompt bronchoconstriction with high phosgene doses (1, 28). 2) Carbon black suspension was poured under constant pressure into the tracheae of anesthetized freshly-killed rats, normal or immediately post-gassing, and the lungs

examined histologically. The distribution of particles in the gassed as compared with normal lungs was markedly irregular (fig. 2), which does suggest patchy narrowing of the air tubes. 3) Barium sulphate powder was inhaled by anesthetized dogs, the animals then X-rayed, heavily gassed by tracheal intubation, and again X-rayed. Air passage diameters, measured on the plates, showed, if anything, a dilatation after gassing (table 5).

Little edema fluid drains from the trachea until a dog is in extremis, at which time it may flow copiously from the mouth and nose. This can hardly result simply

TABLE 2. EFFECT OF GASSING AND VAGOTOMY ON TIDAL AIR AND RESPIRATORY RATE

BEFORE EXPOSURE				TIME AFTER EXPOSURE									
No vagotomy													
T.A.	R.R.	2 hr.		3 hr.		4 hr.		6 hr.		8 hr.		10 hr.	
		T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.
64	25	12	70	14	87	11	75	13	64	13	51	14	39
58	23	10	34	18	39	25	35	27	41	19	47		
57	17	15	63	20	71	20	72	17	84	16	75	20	61
55	16	22	24	18	26								
45	24	12	90										
100	12	14	55	16	51								
Vagotomy after exposure													
		1 hr.		2 hr.		3 hr.		4 hr.					
		T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.
60	9	30	124	152	20	184	10	135	12				
89	38	28	135	59	60	79	38	95	38				
Vagotomy before exposure													
Before vagotomy		After vagotomy		1 hr.		2 hr.		3 hr.		4 hr.		6 hr.	
T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.	T.A.	R.R.
36	27	105	9	83	24	89	20						
134	32	171	11	212	12			290	16	340	21		

from diaphragmatic or costal pressure on the lungs, since positive pressure pneumothorax will not produce such drainage (16). It thus suggests a terminal bronchiolar dilatation.

The passive factor in bronchial narrowing is difficult to evaluate. Peribronchial edema, mucosal necrosis with sloughing, and excessive mucous secretion into the lumina do occur and undoubtedly contribute to respiratory obstruction.

Despite the conflicting findings, we feel that bronchial narrowing of varying intensity and duration, with partial functional obstruction leading to emphysema, does occur promptly after gassing. Whether this narrowing is primarily passive or primarily muscular is not clear. Whether, in addition to bronchioles, alveoli are directly damaged will be considered later.

*Massive Exposures.* Animals may die after exposures to high concentrations of phosgene without pulmonary edema, and it has been argued from this and from the pathological changes in the brain, kidney and liver that phosgene per se acts on extrapulmonic tissues (14). We, too, have found it possible to kill animals with very high phosgene concentrations (up to saturation levels) without pulmonary edema, but with pathological changes entirely different from those usually seen. Some of these were also studied during the First World War (1, 29). In direct contact with blood, phosgene causes hemolysis and acid hematin formation, and these changes can be seen to occur within the lung of an animal exposed to sufficiently high concentrations. Such lungs, though edema free and well aerated, feel more solid than normally. A cut surface shows a strongly acid reaction to litmus, even to the pleura.

TABLE 3. INTRATHORACIC PRESSURE BEFORE AND AFTER GASSING (DOGS)

All figures in mm. H<sub>2</sub>O

	DOG 1				DOG 2			
	Right side		Left side		Right side		Left side	
	insp.	exp.	insp.	exp.	insp.	exp.	insp.	exp.
2 hr. before.....	-46	-30	-40	-20	-68	-58	-90	-30
1 hr. before.....	-50	-40	-48	-36	-70	-54	-60	-40
0.5 hr. after.....	-60	-30	-50	-30	-140	0	-90	-10
2 hr. after.....	-120	-40	-100	-20	-140	-40	-60	0
3.5 hr. after.....	-140	+100	-110	0	-110	-30	-110	-10
5 hr. after.....					-140	-20	-90	0
<i>Averages</i>								
	Insp.		Exp.					
Pre-gassing.....	-59		-39					
Post-gassing.....	-107		+24					
Change.....	-48		+63					

Gross areas of brownish discoloration are found microscopically to contain dilated capillaries plugged with hemolyzed erythrocytes. Sudden death in such cases is undoubtedly due to this local tissue destruction and interrupted circulation. The HCl liberated denatures protein, lyses cells, forms hematin and leads to widespread capillary obstruction and asphyxia. On direct *in vivo* microscopic visualization of the frog lung, during exposure to massive doses, the capillary circulation is seen to come to a standstill as the vessels become plugged with hemolyzed and hemolyzing cells. In no case, of many hundreds of normal exposures in this laboratory, has death been seen without pulmonary edema unless such very high concentrations were used.

We cannot explain Rothlin's findings (14) of death without pulmonary edema after exposure to moderately high concentrations and without widespread necrosis. In our experience, death follows gross pulmonary edema or, with extreme exposure, widespread pulmonary circulatory arrest, or some combination of these.

*Influence of Respiratory Epithelium.* If in mammalian alveoli there is no epithelium covering the capillary endothelium (30, 31), then it is only in the lung, of all the avenues of entry tried, that phosgene or diphosgene can come into direct contact with capillary endothelium without having to pass another cellular layer. Since hydrolysis is extremely rapid (31a), the agent might never reach peritoneal blood vessels covered by mesothelium, in harmony with the innocuousness of intraperitoneal injection. In line with this argument, we have found that goldfish, which have a gill epithelium, and frogs, which do have a lung epithelium, can tolerate high concentrations (frogs up to at least 2.8 mgm/liter for 10 min.). On the other hand, birds, which

TABLE 4. EFFECT OF DIPHOSGENE ON RATE OF TRANSUDATION OF TRACHEALLY INJECTED RINGER THROUGH VISCERAL PLEURA

PERFUSION FLUID	AGENT	AMOUNT	FLOW RATE	
			Before	After
		cc.	cc/min.	cc/min.
Lockes	pilocarpine	1 of 1:1000	1.0	0.2
Lockes	epinephrine	1 of 1:1000	0.2	1.2
Lockes	pilocarpine	1 of 1:1000	1.2	0.45
Lockes	atropine	1 of 1:1000	0.45	1.15
Lockes	diphosgene	0.01	1.0	0-0.8
Lockes	diphosgene	0.01	0.8	0-1.0
Lockes	diphosgene	0.01	1.0	0-1.2
Lockes	diphosgene	0.01	1.2	0-1.0
Lockes	HCl (conc.)	0.01	1.2	0.0
Lockes	HCl (conc.)	0.01	1.0	0.7
Lockes	HCl (conc.)	0.01	0.8	0.6
Lockes	HCl (conc.)	0.02	1.2	0.8
Lockes + NaHCO <sub>3</sub>	HCl (conc.)	0.2	0.6	0-0.6
Lockes (pH 8.0)	HCl (conc.)	0.1	1.4	1.4
Oil	diphosgene	0.01	1.0	0.8
Oil	diphosgene	0.01	0.8	1.6
Oil	diphosgene	0.01	1.6	2.0
Oil	diphosgene	0.01	2.0	1.4

do not have a pulmonary epithelium covering the blood vessels, are said (32) to be killed by the gas at moderate concentrations (1 mg/liter for 30 min.).

Such experiments are at best suggestive, since the actual exposure of the absorbing surface and the amount of toxic agent actually retained by these various animals is unknown. Indeed, only recently (33) has a method been developed for measuring the retained dose of the toxic agent in mammals (21).

#### DISCUSSION

Gassing one lung of a pair, using the other as a control, seems to provide an unequivocal experiment. The fact that only the exposed lung of a pair shows damage, its protected mate and other tissues remaining normal, is very strong evidence that the inhaled diphosgene per se acts locally and only locally. This evidence plus the

results of crossed circulation and transfusion experiments shows the absence of specific pneumotoxin in the circulating blood or edema fluid. The presence of limited amounts of capillary permeabilizing factor, histamine and thromboplastic substance is not excluded. Further evidence against direct extra-pulmonic action by the inhaled agent is seen in the absence of damage by parapulmonically administered diphosgene up to doses yielding sufficient HCl to produce local necrosis.

Although damage to the bronchi and bronchioles is clear cut, it is still not certainly established whether or not the alveoli themselves are directly injured. The evidence, pro or con, is equivocal. A direct search for histological signs of damage to the alveolar wall has been generally fruitless. Since thrombosis is a common consequence of blood vessel injury, the finding of thrombi in the alveolar capillaries would argue for alveolar damage. Such thrombosis in dogs, goats and man was often claimed (8, 34-36); but the most specific (9) or recent (23) studies have been negative, despite the finding of an occasional thrombus (18). Fibrin strands crossing the alveolar septa

TABLE 5. EFFECT OF POSITIVE PRESSURE AND DIPHOSGENE ON DIAMETER OF BRONCHI OUTLINED WITH INHALED BISMUTH SUBCARBONATE

CONDITION AT TIME OF X-RAY	DIAMETERS OF A SERIES OF AIR TUBES												
<i>Dog 1</i>													
15 mins. before gas.....	0.9	0.9	1.0	1.3	1.5	2.0	3.3	3.4	5.0	5.5	8.0	12.5	13.0
Just before gas.....	0.8	0.7	1.1	1.2	1.4	1.9	3.6	3.3	5.0	6.0	7.8	13.0	12.9
10 mins. after gas.....	0.9	0.8	1.1	1.0	1.3	1.8	3.8	4.9	6.5	6.3	10.1	10.9	13.8
20 mins. after gas.....	0.9	0.9	1.1	1.0	1.6	1.6	3.8	5.2	6.7	7.0	9.5	13.5	13.4
Pos. press. after gas.....	1.0	1.0	1.0	1.0	1.9	1.6	4.8	6.8	8.4	8.1	11.0	15.0	14.8
45 mins. after gas.....	0.8	0.9	1.1	1.0	1.2	1.9	3.8			6.5	9.2	13.6	13.0
Rt. vagus stim. 70 mins. after gas.....	0.8	0.9	1.1	1.0	1.7	1.8	3.6			6.5	8.9	14.4	12.4
<i>Dog 2</i>													
Before gas.....						1.8	3.7				8.5	11.5	12.6
Immed. after gas.....						2.0	4.3				9.9	11.0	13.0
Immed. after repeat gassing.....						2.8	4.5				9.2	11.0	13.0
50 mins. after gas.....						2.0	4.5				10.0	10.9	12.6

N.B. All X-ray exposures made during the expiratory phase of respiration.

were taken as evidence of damage (9), but the strands might be passing through inter-alveolar pores, the existence of which has now been fairly definitely established (37). A decrease in the number of mitochondria in the alveolar endothelium has been reported (38), but this is hardly decisive.

Dilation and engorgement of alveolar capillaries appears only after the development of emphysema and bronchiolar changes, and alveolar edema is seen only after peribronchial edema is established. It does not follow, however, that alveolar damage is late or secondary. Pulmonary lymphatics do not extend beyond the alveolar ducts; there are none in the alveolar walls proper (39). Pulmonary lymph flow is markedly increased after phosgene exposure, but even so the overloaded lymphatics are able to handle only some 10 per cent of the total edema fluid produced (40). Edema fluid might, then, be first produced in the alveoli, but, being carried off along the septa to the ductule lymphatics, initially escape detection. The increased lymph flow, exaggerated by dyspnoeic movements (41) and by hypoxia, leads to peribronchial edema which only later backs up into the alveoli. Thus, peribronchiolar edema might be seen before alveolar edema even though the edema fluid originated as a result of

alveolar capillary leakage. This would be excluded if the finding of Coman *et al.* (18), that the lymphatics are not engorged at the time when peribronchiolar edema is seen, is confirmed with techniques for staining protein poor transudates.

#### SUMMARY

Experiments were designed to discover the locus or loci of action of inhaled phosgene and diphosgene.

Evidence from unilateral gassing, crossed circulation and transfusion experiments and the results of parapulmonic administration of the toxic agent, show that the inhaled substance per se acts only in the lung. Pathological changes in other organs are secondary to this. Action on the lung is probably direct; there is no evidence for a circulating pneumotoxin or other toxic substance. The damaged lung may, however, liberate thromboplastin and capillary permeabilizing substances into the edema fluid.

Bronchioles are certainly injured, with some degree of inflammatory response, necrosis or scarring. Bronchiolar narrowing of variable severity and duration occurs shortly after exposure, and may involve both active bronchoconstriction and passive narrowing due to the mural inflammation and edema. It can account for the early emphysema.

There is no unequivocal evidence of damage to the alveoli. Although peribronchiolar perivascular edema precedes alveolar edema, such a sequence could follow prior damage to alveolar capillaries.

Massive doses of the toxic agent produce pulmonary damage which is qualitatively different from that following more moderate, though still 100 per cent lethal, doses. Death after moderate doses is due to anoxia developing gradually with pulmonary edema; death after massive doses is immediate, due to occlusion of the pulmonary circulation from intravascular clotting and acid hemolysis.

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# EFFECT OF VARIATIONS IN NUTRITIVE DENSITY ON INTAKE OF FOOD OF DOGS AND RATS

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THE animal's ability to regulate its food intake in accordance with its caloric needs despite variations in energy output is a commonplace one, although the mechanisms involved in such physiological regulations are poorly understood. It is likewise apparent that the animal's ability to adjust food intake to changes in the caloric concentration or nutritive density of its foodstuffs is a prerequisite for such regulation. Indeed, it is widely held that animals *do* adjust their food intake in keeping with changes in the caloric concentration of their diets with a high degree of precision (1). What is remarkable however is the scantiness of the documentation upon which such a comprehensive statement rests. Cowgill in 1928 studied the effect of increasing the energy content of the diet on food intake in four dogs. Under the conditions of his experiment the dogs "ate fewer grams of the ration but approximately the same number of calories per day as formerly" (2). Adolph (3) has more recently presented evidence on the effects of dilution of diet with inert material on food intake in rats.

Because further observations on this point seemed necessary to us in the course of a systematic analysis of the factors which might possibly be concerned in the regulation of food intake in accordance with bodily need, and especially on the rate at which such adjustments are made, we undertook to study the effects of variations in nutritive density on food intake in the dog over prolonged periods of time by the progressive dilution and concentration of its diet. As a companion study, and because we were struck with the reported rapidity with which these adjustments were made in the rat, similar experiments on the progressive dilution and concentration of its diet were also performed in the rat.

## METHODS

*Dogs.* Six healthy mongrel dogs were employed in this study. The animals were housed in individual cages, in an air-conditioned, relatively constant temperature room. They were fed once daily at the same time, and by the same individual throughout this study. Excess amounts of food were offered to the dogs in tared pans and allowed to remain in the cages for exactly 45 minutes, following which they were removed and weighed. The dogs were weighed weekly. There was free access to water at all times. These animals had been used in previous feeding experiments, and were stabilized as to food intake and weight.

During *period 1* the animals were offered a diet of a dried commercial dog food (Pard, dehydrated, Swift) moistened with cooked meat juice, for 10 weeks. One gram of this mixture was equivalent to 2.5 calories; the moisture content was 40 per cent.

During *period 2* the animals were offered a diet of a complete commercial dog food (Pard, regular, Swift); one gram of this was equivalent to 1.2 calories; the moisture content was 70 per cent. Thus the diets of *periods 1* and *2* differed essentially only in moisture content and the absence of meat juice in the diet of *period 2*; the caloric value of the dry diets was the same for these two periods. This diet was continued for from 7 to 11 weeks, until the individual dogs had stabilized either the daily average food intake or the weekly weight or both.

During *period 3*, the diet consisted of the complete commercial dog food of *period 2* diluted with cellulose (Ruffex, Fischer) and water so that the resulting mixture was similar to the diet of *period 2* in consistency and moisture content (70 per cent), but one gram of this mixture was equivalent to 0.7 calories. This diet was continued for from 11 to 15 weeks, until the individual dog had stabilized either his average daily food intake or his weight or both.

During *period 4*, the animals were returned to the complete dog food of *period 2* for 10 weeks. One gram of this diet was equivalent to 1.2 calories.

*Rats.* Adult male white rats grown in this laboratory were housed in individual cages in a relatively constant temperature room, with free access to water at all times. The control diet was an adequate commercial dry feed (Purina, Chow) one gram of which was equivalent to 3.3 calories. The diet was supplied in small glass jars placed within deeper jars to hold scattering to a minimum. The food intake and body weight were recorded daily.

Groups of 5 rats each, after control periods of 10 days, were placed on a diet composed of this dry feed thoroughly mixed with varying proportions of cellulose (CellufLOUR, Chicago Dietetic) amounting to 25 per cent, 35 per cent, 50 per cent, and 75 per cent of the diet.

After 8 to 11 days, the animals on the first three of these dilutions were returned to the control diet for 10 days.

## RESULTS

*Dogs.* The individual responses of the 6 dogs to the variations in nutritive density of the diet are presented graphically in figures 1 to 6. The average intake of food is calculated in terms of the weekly average of daily intake. The calculation of the calories consumed per kilogram of body weight are based on the weekly body weight; total solids consumed were calculated on the basis of the moisture content of the diets as given above.

It will be seen that all dogs made some adjustment in food intake in keeping with the variations in nutritive density of the diet. However, such adjustments did not occur in every dog with every variation; there were marked individual differences between dogs in the rates at which such adjustments were made, in the maintenance of these adjustments, and in the precision with which caloric need was balanced during the various dietary periods.

Compared with their food and caloric intake of *period 1*, 5 of the dogs during *period 2* tended to increase the quantity of food consumed, while one dog (No. 15) made no such increase. Of the 5, the increase was sustained in only 3. Further, 4 of the dogs making adjustments of food intake required between 5 to 8 weeks to reach their maximum intake; one required 2 weeks (*dog 18*) but had an unsustained increase. In no dog did the increased volume of food consumed compensate completely for its lowered caloric value. Weight loss occurred in 5 dogs during this period. All dogs tended to ingest smaller amounts of solids as compared with the control period.

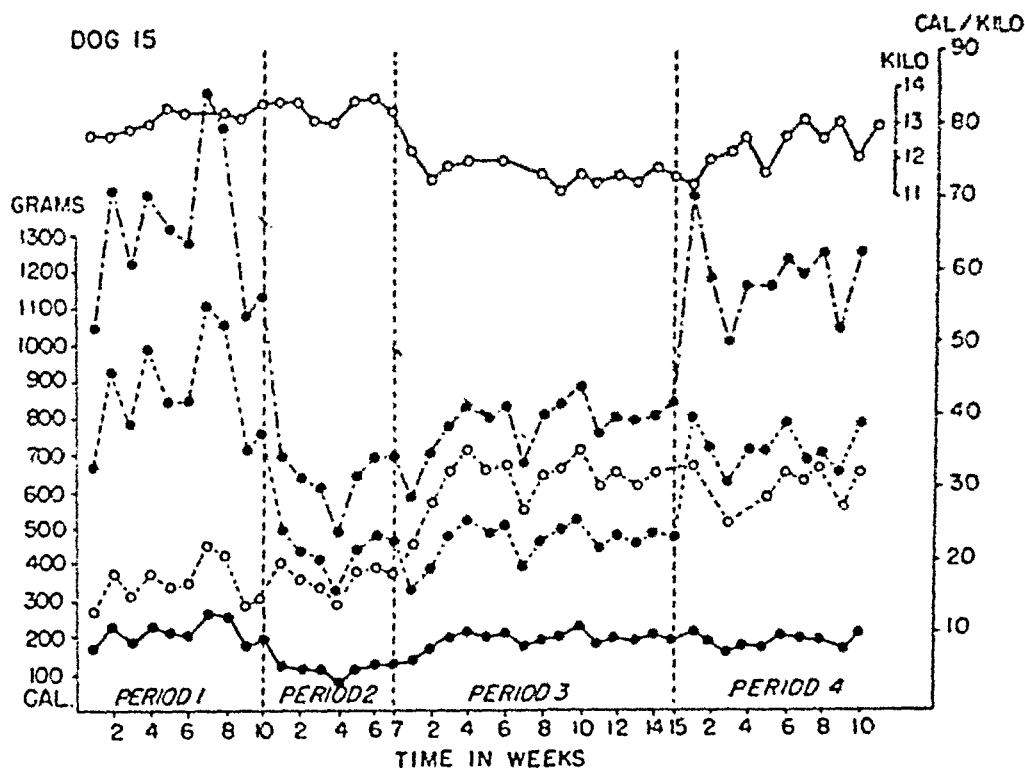


Fig. 1. ○---○ Grams of Food Consumed    ●---● Total Calories Consumed    ○—○ Body Weight    ●—● Calories/Kilo Body Weight    ●—● Grams of Solids Consumed

During *period 3*, all dogs increased the total volume of food and amount of solids eaten in keeping with the further lowering of the caloric value of the diet, requiring from 4 to 10 weeks to reach their maximum intake. In no dog did the increased volume of food consumed compensate for the diminished caloric value of the diet when compared to *period 1*; in 2 dogs (*dogs 17, 18*) the increased volume of food consumed did not compensate for its diminished caloric value when compared to *period 2*; further weight loss occurred in these dogs, as well as in 2 others (*dogs 15, 16*). The increase in food consumed was sustained in only 4 of the 6 animals.

In *period 4*, with the return of the undiluted diet, there was a sudden transient increase of intake in 2 dogs (Nos. 17, 18).

In keeping with the increased caloric value of the diet in this period, volume of food and amount of solids eaten were diminished in only 3 of the 6 animals (*dogs 16, 17, 20*), which required 3 to 4 weeks in each case to reach the minimum amount. In

only two instances (*dogs 17, 20*) did the diminished intake of food compensate for its increased caloric value as compared with *period 2*, and in only one (*dog 20*) as compared with *period 3*. All animals restored their losses in body weight during this period.

*Rats.* The results of these experiments are summarized in table 1. It will be seen that in general some compensatory changes in the volume of food consumed occurred with changes in concentration of calories in the diet. However, sustained adjustment did not occur at every dilution. Differences occurred in the rate and the success with which caloric needs were balanced.

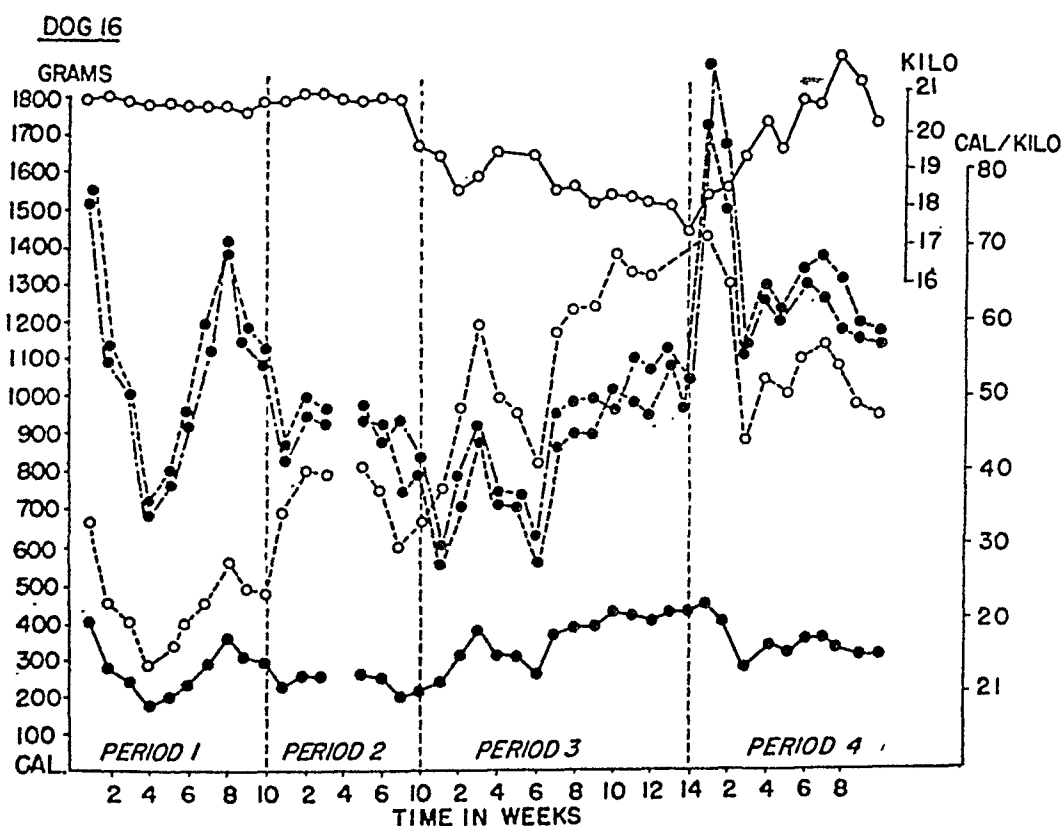


Fig. 2. See key, fig. 1.

From table 1, it will be seen that on the average the amount of food eaten increased over the control period in all dilutions. The maximum food intake occurred on the 7th day on the 25 per cent diluted diet, the 1st day on the 35 per cent diluted diet, the 5th day on the 50 per cent diluted diet, and the 7th for the 65 per cent diluted diet. From the averages it will be seen that the increased volumes consumed were not uniformly maintained. On the average only at 25 per cent dilution did the increased volumes of food consumed compensate for the lowered caloric values of the diet offered and even here initial weight loss occurred; caloric deficits were present in all other dilutions and corresponding weight loss occurred. Sixty-five per cent dilution offered the greatest stimulus to increased food intake; there was decreased effectiveness at the 50 and 35 per cent dilution level.

When the rats were returned to the control diet after 8 to 11 day periods on

diluted food there was no striking immediate change in the amount of food eaten in any of the dilutions tested (25, 35 and 50%).

### DISCUSSION

It is clear that under the conditions of these experiments variation in the caloric concentration of the diet does not lead to an immediate and completely compensatory adjustment in the quantity of food consumed.

With progressive dilutions and concentration of the diet of the dog in three consecutive steps, of 6 dogs studied, 2 made essentially no readjustments at two of these steps, and one dog made none at one of these steps. In those instances where the

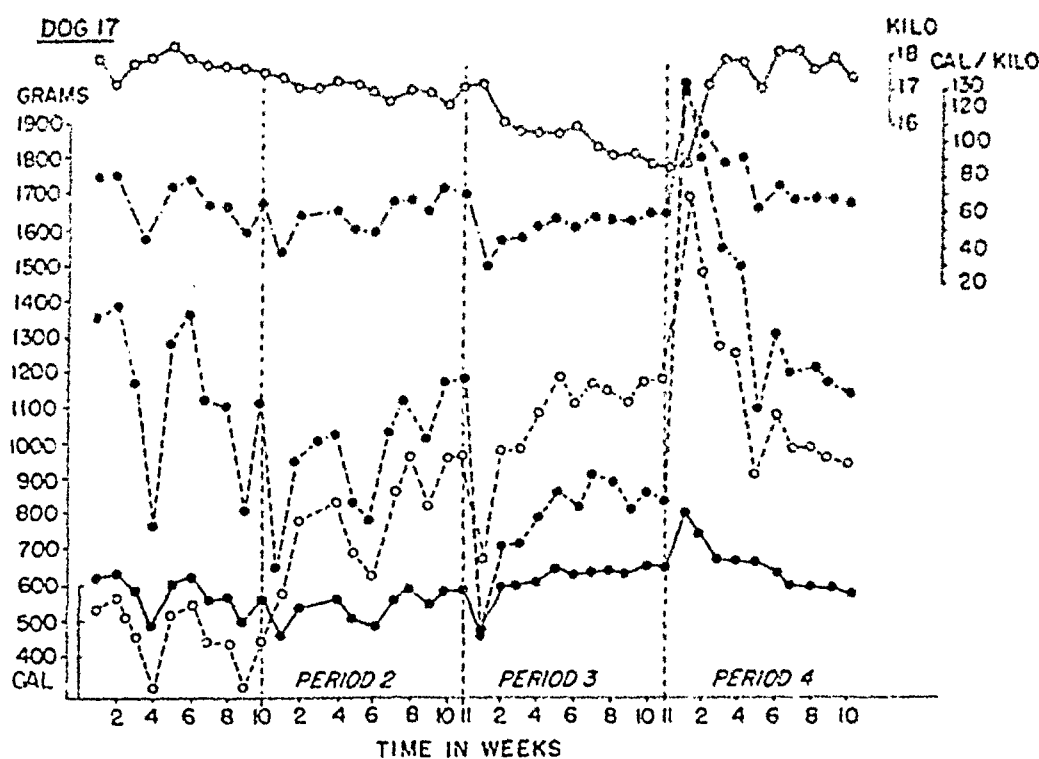


Fig. 3. See key, fig. 1.

quantity of food eaten did vary with the nutritive density of the diet offered the dog, the changes were manifested extremely slowly, and required from weeks to months to become fully developed. The gradualness of such responses is further emphasized when the day-to-day variations are considered as well as the weekly averages of food intake. When such compensatory increases in food eaten were made, they were frequently transient, or only partially sustained. If the calories ingested are compared to the control period it will be seen that during the periods of dilution, the increased volume of food consumed did not compensate calorically for the lowering of the nutritive density of the diet.

From these results it would appear that the dog achieves satiety in part by the ingestion of an appropriate volume of food, and manifests a strong tendency to continue ingesting this same volume of food daily. This 'inertia' is emphasized by the

slowness with which the physiological mechanisms concerned in adjusting food intake to caloric need respond to the alterations in nutritive density of the food available to the animal.

However, this persistence in ingesting the same volume of food daily is of course not absolute, and the dogs did respond after variable periods of time to the varied degrees of dilution by increasing the quantity of food eaten. In this series of experiments a 65 per cent reduction in the caloric content in the diet stimulated increased food intake in all dogs.

In considering the possible factors which are operative in modifying the dog's tendency to go on eating a given volume of food daily, it is clear that they are not directly or immediately related solely to the nutritive density of the diet, since these

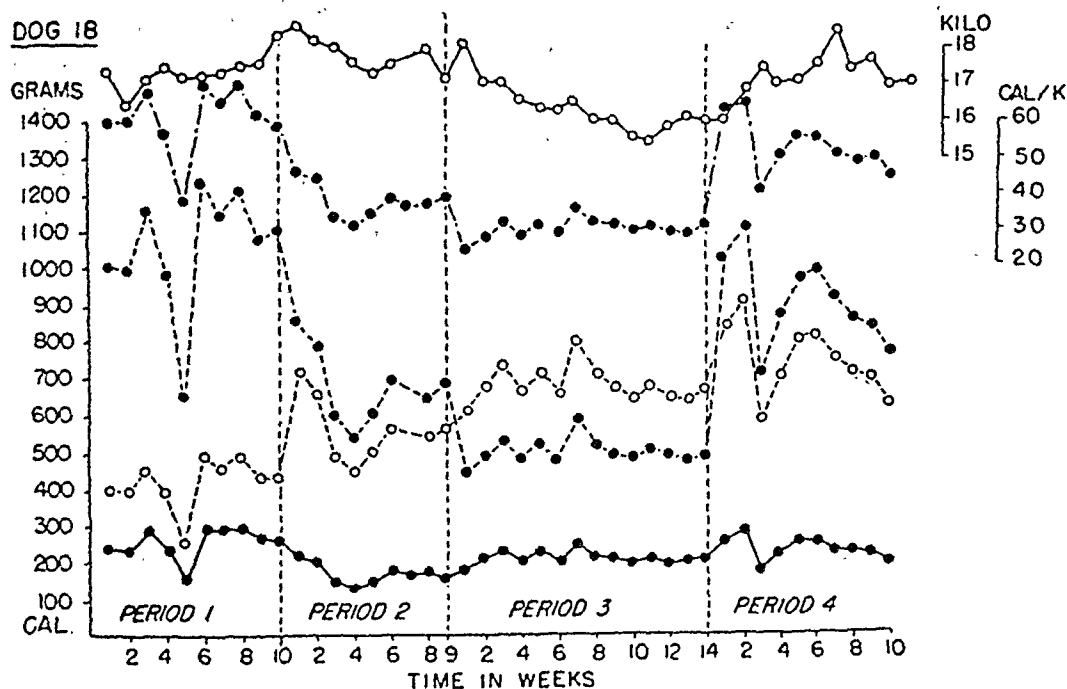


Fig. 4. See key, fig. 1.

hypothetical factors require long periods of time to become operative. Because of this lag in adjusting caloric intake, caloric deficits are developed, and these may possibly have an intermediate rôle. From our previous studies we have obtained some evidence that caloric deficits in both dog and rat are not balanced from meal to meal, or from day to day in short term experiments (4), and the time relationships in the present studies are consistent with this interpretation. The adjustments in food intake in the direction of balancing these deficits occur in terms of weeks and months rather than of days.

That the effects of caloric deficits may be operative at slow rates as a part of some intermediary mechanism for adjusting food intake to caloric needs is indicated by the fact that the maximal attempts at compensation tended as a rule to occur at the periods of greatest loss of body weight, although not in every case.

These experiments do not furnish any evidence on how such mechanisms might

operate. As a theoretical possibility, Harris (5) hypothesized that general states of well being are associated with learned responses to various types of diet. But even these tentative remarks must be qualified by the fact that weight loss did not occur at a uniform rate in keeping with the caloric deficits, and that the volume of food consumed did not vary directly with the body weight. If the percentage increase in average amount of food eaten per day during *period 3* as compared to *period 2* is plotted against the per centage loss of body weight of these periods, there is no uniform pattern for all the dogs. Loss of weight per se does not appear to play the predominant rôle in increasing food intake.

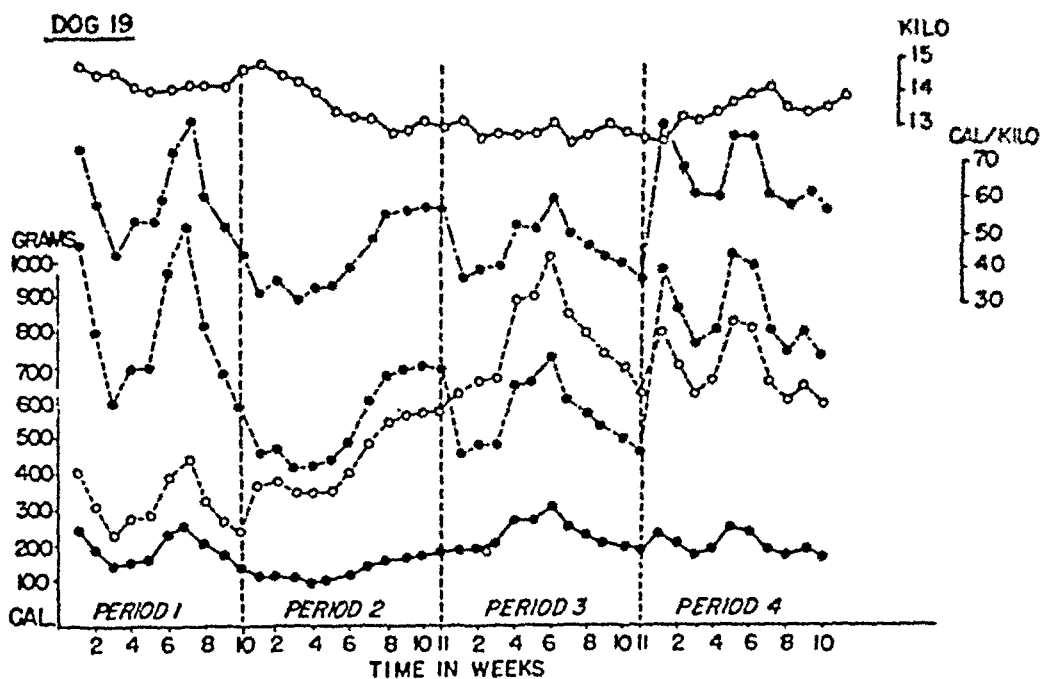


Fig. 5. See key, fig. 1.

Although the interpretation advanced in the present report is at variance with that of Cowgill (2), the results of that observer indicated that when 4 dogs were transferred from a diet of 4.88 calories per gram of food to one containing 6.06 or 6.1 calories per gram of food all animals adjusted to the higher calorie ration by a gain in body weight (average 0.47 kilos). Cowgill's suggestion that this perhaps represented the 'greater palatability of such a diet' diminishes the force of his argument for the predominant rôle which he ascribed to energy requirements in determining food intake. Furthermore, one of his 4 dogs responded to the change in diet by greatly increasing the number of calories per kilogram of body weight ingested per day. In the present series of experiments it will be seen from figures 1 to 6, that after the first 3 weeks of *period 4*, three of the 6 dogs continued with an increased number of calories consumed per kilogram of body weight per day. We would interpret this as illustrating the variety of individual responses.

In the rat our findings are in general agreement with those reported by Adolph (3). He observed that not until diets were diluted as much as 50 per cent were they

insufficient to promote growth as measured by body weight. In our hands diets diluted more than 25 per cent were not able to maintain body weight if the diets were fed for periods longer than 3 days.

We observed as did Adolph that a return to a more concentrated diet did not lead to the intake of extra volumes of food when weight losses had developed as the result of depletion. If this is considered in connection with the evidence that complete deprivation of food in the rat for periods of 1 to 6 days (3) does not furnish a stimulus for increased intake, it becomes increasingly clear that the rat manifests a

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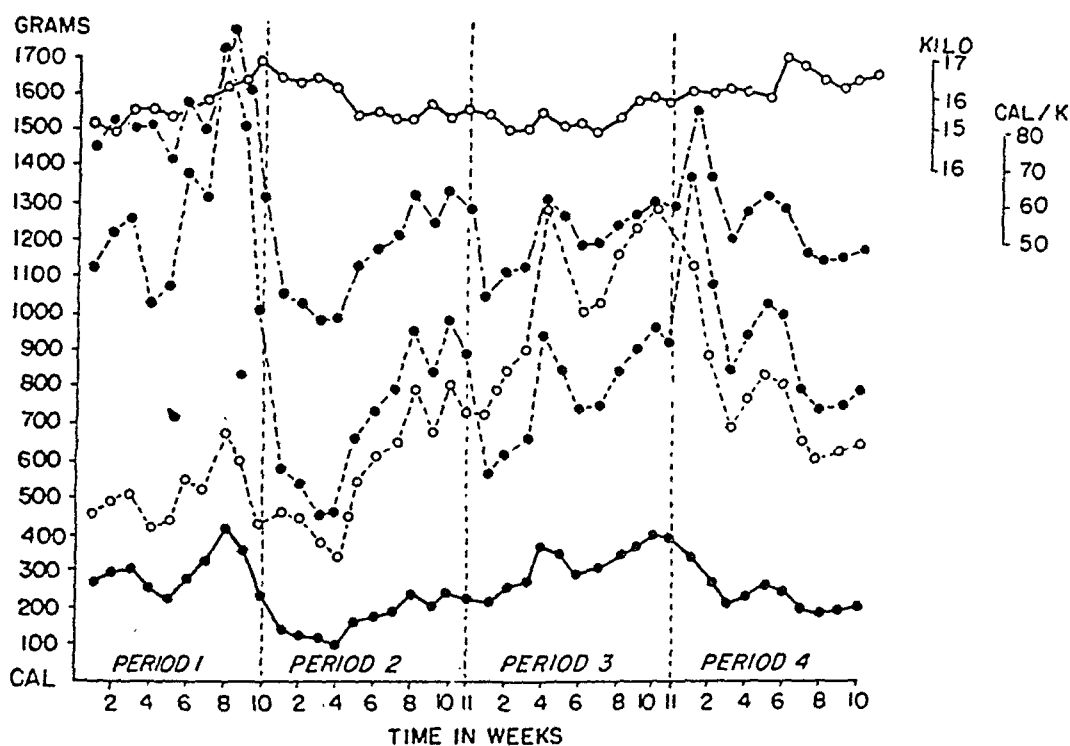


Fig. 6. See key, fig. 1.

tendency to continue ingesting the same volume of food daily, despite caloric deficits or excesses.

As in the dog, this tendency toward the persistence of uniform average daily volume intake is modified by the tendency to maintain caloric equilibrium. In experiments of this type in which an inert diluent is used, it must be borne in mind that the flavors of the diet are being diluted *pari passu* with the calories. The factor of flavor probably played a rôle in the transition from the diets employed in *period 1* where meat juice was used to *period 2* where it was absent. That palatability of the diet furnished may play a rôle is also suggested by the fact that the maintenance of body weight did occur after some days on the diet diluted only 25 per cent, but not on those diluted further. Yet even in the case of the 25 per cent dilution maximum adjustment required 7 days to be reached.

These rats on a diet of 25 per cent dilution lost weight initially. This finding emphasizes the failure to make rapid caloric adjustment even in those cases where

ultimate adjustment is made. From the maximum volumes of food that the rat is found to be capable of eating on the 65 per cent dilution it is clear that the failures in the caloric adjustment at dilutions such as 35 and 50 per cent are not due to limitations in the physical capacity of the rat to ingest sufficient amounts of food. Unlike the dog, the number of calories per gram of body weight consumed by the rat did not vary more than 10 per cent during all test periods.

TABLE 1.—EFFECT OF DILUTION OF DIET ON FOOD INTAKE IN RATS

PROCEDURE	DAY	65% DILUTION			50% DILUTION			35% DILUTION			25% DILUTION		
		Solids gm/day	Cal. con- sumed	Body Wt.	Solids gm/day	Cal. Con- sumed	Body Wt.	Solids gm/day	Cal. Con- sumed	Body Wt.	Solids gm/day	Cal. Con- sumed	Body Wt.
Control diet	10 days	15	49.5	276	18.9	62.4	332	18.6	61.4	378	18.2	59.4	347
Diluted diet	1	14.4	22.2	275	21.0	34.6	331	22.0	48.4	374	22.8	57.0	343
	2	13.8	16.6	259	23.6	38.9	330	21.2	46.6	374	23.5	58.8	341
	3	25.2	30.0	283	25.0	41.3	322	21.2	46.6	367	21.7	54.3	338
	4	27.2	32.4	249	24.2	39.9	321	17.8	39.2	363	24.0	60.0	340
	5	34.0	40.8	248	27.1	44.7	322	18.0	39.6	363	19.8	50.0	340
	6	38.2	45.8	244	23.0	37.9	320	17.8	39.2	360	22.5	56.2	345
	7	36.6	43.9	244	23.1	38.1	321	16.4	36.1	358	24.3	60.8	347
	8	33.2	39.8	242	20.0	33.0	317	18.6	40.9	356	23.8	59.5	348
	9	33.6	40.3	244				20.8	45.8	353	23.2	58.0	346
	10	30.4	36.5	243				16.2	35.6	345	23.7	58.3	349
	11							16.6	36.5	343			
Control diet	1				21.5	70.9	327	21.4	70.6	355	21.8	71.9	350
	2				22.0	72.6	328	17.8	58.9	358	20.8	68.6	351
	3				22.0	72.6	328	19.6	64.7	360	22.4	73.9	353
	4				20.6	67.9	329	19.4	64.0	360	19.2	63.4	350
	5				23.8	78.5	335	17.8	58.7	361	18.4	60.7	352
	6				16.8	55.4	334	19.4	64.0	359	19.8	65.3	353
	7				17.0	56.1	335	16.6	54.8	362	17.6	58.8	354
	8				18.4	60.7	332	18.0	59.4	363	18.2	60.0	355
	9				16.0	52.8	335	20.0	66.0	367	19.2	63.4	355
	10				18.4	60.7	338	19.8	65.4	368	17.8	58.7	356

Each value represents the average of 5 rats.

The complexity of the responses of these two species, which was rightly stressed by Adolph, indicates to us that multiple factors are operative and that the recent statement, "The primary urge in the ingestion of food is the satisfaction of energy requirements" (6), does not do full justice to other components of the regulatory mechanisms involved.

#### SUMMARY AND CONCLUSIONS

The intake of food in the dog and rat was measured under conditions in which the nutritive density of the diet was varied. Although compensatory adjustments in

food intake were made in keeping with the caloric value of the available diet, marked individual differences were observed in the rate and precision with which caloric adjustments were made. The tendency to ingest a constant average daily volume of food, 'eating for bulk', was modified only slowly and incompletely by the tendency to balance caloric deficits, 'eating for calories'. A factor of palatability is probably also concerned in these adjustments of food intake.

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# SECRETION OF GASTRIC MUCIN IN RESPONSE TO SHAM-FEEDING AND HISTAMINE STIMULATION<sup>1</sup>

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THERE is considerable evidence that a mucoprotein having mucoitin sulfuric acid as its prosthetic group is as much a major constituent of gastric juice as are hydrochloric acid and pepsin (1-3). Since this mucoprotein is present in the juice in solution, it has been termed 'dissolved mucin' (1). Another mucoprotein (2) has been found in alkaline or neutral mucus secreted spontaneously by the stomach in fasting animals, the elementary composition and physical properties of which are distinctly different from those of 'dissolved mucin' (2). That the gastric mucosa may secrete at least two different mucins is also indicated by the isolation of two complex carbohydrates from 'Wilson's Mucin' obtained from the gastric mucosa of pigs (4). The mechanism of secretion of these mucoproteins has been the subject of an intensive study carried on principally in Babkin's laboratory. The results obtained there were interpreted (1c) as indicating that: 1) "There is strong evidence that the concentration of 'dissolved mucin' increases when the vagi are stimulated"; and 2) "Histamine inhibits the discharge of pepsin and presumably mucin." However, this evidence was not regarded as conclusive (1c), since reliable methods for the determination of mucin were unknown and those employed "could give at best only approximate results."

It was obvious that the problem had to be re-investigated as soon as more dependable chemical methods for mucin determination were available. We have succeeded recently in developing a reliable method for the determination of 'dissolved mucin' in gastric juice (5) and in an attempt to gather further information on the mechanism of mucin secretion, we have applied this method to the study of its secretion in response to sham-feeding and to histamine.

## METHODS

Most of the experiments were performed on 3 dogs with gastric fistula and esophagotomy. The animals were fasted for 18 to 20 hours but water was given *ad lib*. The stomach was usually empty and clean at the beginning of each experiment and only typical alkaline mucus was secreted during the control period. The effect of sham-feeding with meat for 3 or 5 minutes was studied in 20 experiments; in some, sham-feeding was repeated and 'conditioned stimuli', such as the sight of food and other procedures associated with the process of eating, were also employed. The effect of histamine was investigated in 4 experiments on 2 esophagotomized dogs and

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in acute experiments on 7 dogs. In the former, histamine was administered after preliminary atropinization in order to eliminate possible interference by incidental psychic influences transmitted through the vagus nerve.

The technique used in acute experiments was as follows: Deep anesthesia was induced by intravenous injection of sodium pentobarbital. A tracheotomy was performed; the external jugular vein was cannulated and the esophagus was ligated in the neck. A gastric fistula was sutured through the abdominal incision and the pylorus was ligated. The dogs remained in a supine position for 2 or 3 hours to allow operative shock to subside, after which they were suspended in a prone position and the actual experiment was started. Saline was injected intravenously at 30-minute periods in amounts necessary to compensate for the loss of body fluids (in the gastric and urinary secretions) during the course of the experiment.

Mucin was determined by estimating the uronic acid liberated from the protein material of gastric secretion by the naphtho-resorcinol method which has been described previously (5), and in several of the experiments Dische's carbazole method (6) for hexuronic acid was also used. Results obtained by both procedures were in satisfactory agreement. Results were calculated for mucin assuming that the uronic acid content in 'dissolved mucin' is 1.22 per cent as reported previously (5). The specimens were also analyzed for pepsin, total chloride and total acidity and the hydrogen ion concentration was calculated from the  $pH$  values determined with the glass electrode.

## RESULTS

*Effect of Sham-feeding and Related Stimuli.* Sham-feeding, whether applied once or repeatedly in the course of an experiment, always resulted in a marked increase in the output of 'dissolved mucin.' A similar effect was also produced by 'conditioned stimuli' related to the act of eating (the sight of the dish used for sham-feeding or noises associated with the procedure). Increase in output of mucin was independent of concomitant changes in mucin concentration. In the experiments started on animals with a resting stomach, only scanty amounts of alkaline or neutral mucus were secreted during the control period. The mucin concentration in this material varied considerably but was always very high. From 17 observations on 2 dogs the mean concentration was 862 mg/100 ml. with standard deviation of 503. After sham-feeding, when the secretion of acid gastric juice starts at a high rate, the concentration of 'dissolved mucin' becomes much lower than in mucus previously secreted. Nevertheless a pronounced increase in the output of mucin is observed (fig. 1 and table 1, part A). If, on the other hand, the cephalic phase of gastric secretion is induced by sham-feeding or other means at a time when the stomach is already secreting acid gastric juice, then both the concentration of mucin as well as its output become higher (fig. 2; also Effect of Second Sham-feeding in fig. 1). Obviously these variations in the mucin concentration are greatly influenced by variable degrees of dilution of the mucoid secretion with the secretion of parietal cells.

Whenever the cephalic phase of gastric secretion was evoked, the output of mucin was increased after each stimulation. Moreover in such experiments the

output and rate of secretion were as a rule correlated (fig. 1). If the rate of secretion can be regarded as a measure of strength of the stimulus, then it would appear that there is a correlation between the output of mucin and the strength of the cephalic secretory impulses.

A very striking feature of all our experiments with sham-feeding was the absence of a quantitative parallelism between the secretion of mucin and pepsin, and particularly between mucin and acid in the course of any single experiment. As a rule the increase in pepsin output after sham-feeding was higher than that of mucin, especially

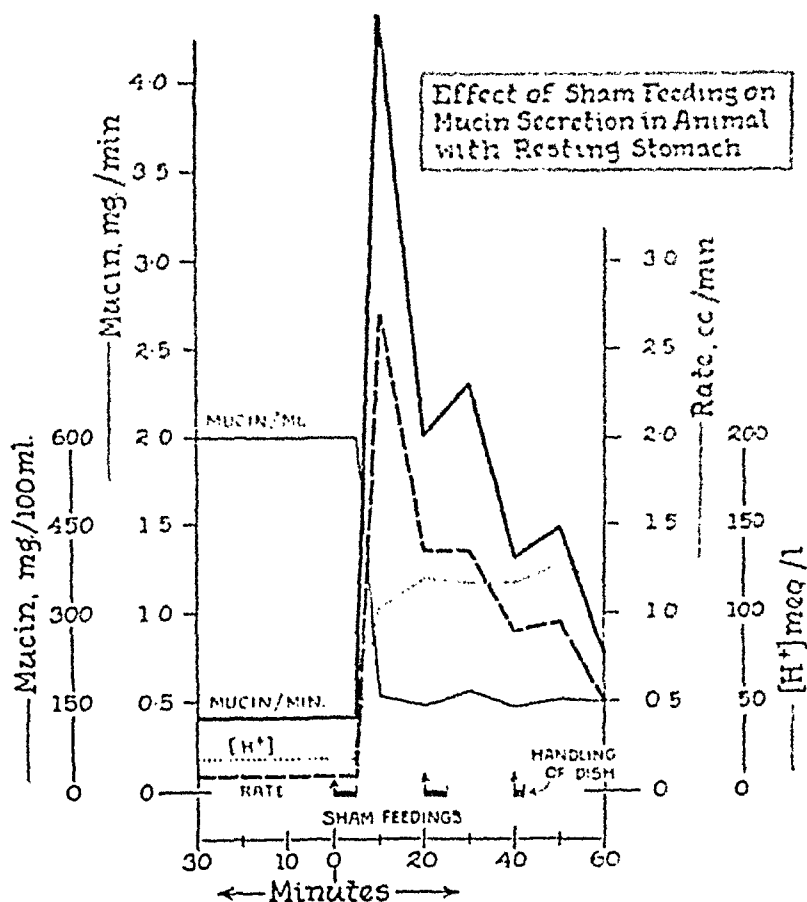


Fig. 1

during the first half hour of sham-feeding, although there were exceptions when the relationship was reversed. During the later stages of secretion there was some tendency for a linear relationship between pepsin and mucin outputs (fig. 2). Since the concentration of both of these constituents of gastric juice was equally affected by dilution with the parietal secretions, the above relationships are reflected in the values for the ratio of their concentrations (table 1). This ratio for the first 30-minute period of secretion after sham-feeding was  $2.07 \pm 0.181$  (standard deviation) and for the second 30-minute period,  $1.04 \pm 0.179$ .

*Effect of Histamine.* Histamine administration in all experiments, chronic and acute, elicited a typical response: a copious secretion of gastric juice characterized by

high acidity and low pepsin concentration. This is illustrated in table 1, part *B* and table 2; the former giving results of experiments on unanesthetized dogs with gastric fistula and esophagotomy; the latter showing results which resemble those of a typical acute experiment in a dog under pentobarbital anesthesia.

The concentration of mucin in gastric juice secreted in response to histamine was consistently lower than the basal values and the concentrations found in the juice secreted on sham-feeding. The output of mucin during the initial period of stimulation lasting for about one hour (table 1, part *B*, specimens 3 to 7, outputs 0.415 to

TABLE 1. SECRETION OF MUCIN IN CHRONIC EXPERIMENTS: RESPONSE TO SHAM-FEEDING AND HISTAMINE PLUS ATROPINE

SPEC.	TIME	RATE	[H +]	TOTAL ACIDITY	CHLORIDE	PEPSIN METT UNITS		MUCIN		P/M
no.	min.	cc/min.	mEq/l.	mEq/l.	mEq/l.	/cc.	/min.	mg/100	mg/min.	ratio
A Sham-feeding	1	30	0.033	+				2100	0.693	
	2	30	0.023					1715	0.395	
	3 <sup>1</sup>	15	0.833	107	127	160	231	164	1.366	1.41
	4	15	0.833	117	140	156	164	190	1.581	.86
	5	15	0.450	117	145	156	185	185	0.834	1.00
	6	15	0.367	123	150	161	231	206	0.756	1.12
	7→	15	0.327	126	159	164	185	188	0.615	.99
	8	15	0.427	112	147	162	185	194	0.829	.95
B Histamine plus atropine	1	30	0.073	96			384	28	274	1.40
	2 <sup>2</sup>	30	0.013							
	3 <sup>3</sup>	15	0.620	100	133	167	125	67	0.415	1.87
	4	15	1.173	141	158	168	35	55	0.645	.64
	5 <sup>4</sup>	15	1.246	141	158	169	9	37	0.461	.24
	6	15	1.460	141	162	167	3	32	0.467	.09
	7	15	0.966	135	160	166	3	25	0.242	.12
	8	15	0.387	129	152	161	+	16	0.062	<.01

Dog C gastric fistula and esophagotomy (16 kg.) ♀.

<sup>1</sup> Animal sham fed 5 minutes at the beginning of this period.

<sup>2</sup> 1 mg. atropine sulfate subcutaneously

<sup>3</sup> 1 mg. histamine subcutaneously

<sup>4</sup> 0.5 mg. histamine subcutaneously

P/M = ratio of pepsin concentration to mucin concentration.

→ Entrance into room of attendant who usually feeds the dog.

0.645 mg/min.) remains within the limits of the basal values ( $0.450 \pm 0.200$  mg/min. for the same dog in 10 observations). In the later stages of the experiment and with the administration of large doses of histamine the output of mucin tends to diminish to levels below the basal values (table 1, part *B*, specimen 8 and table 2, specimens 3, 4 and 5). These findings contrasted sharply with those in the sham-feeding experiments where there was always a pronounced increase in the output of mucin over the control or basal values which lasted as long as the cephalic stimulation was effective.

The secretion of mucin in experiments with histamine was in some respects similar to that of pepsin, since the concentrations of both diminish sharply with the

progress of secretion. However, one significant difference was always observed both in the chronic and acute experiments, namely, that with the progress of secretion after histamine, the fall in concentration of pepsin was always much greater than that of mucin. This is reflected in the sharp decline of the values of the pepsin concentration/mucin concentration ratios which were regularly observed for the consecutive specimens in the course of the experiment (tables 1 and 2).

In general the above results indicate that histamine does not stimulate mucin and in large doses even appears to inhibit it.

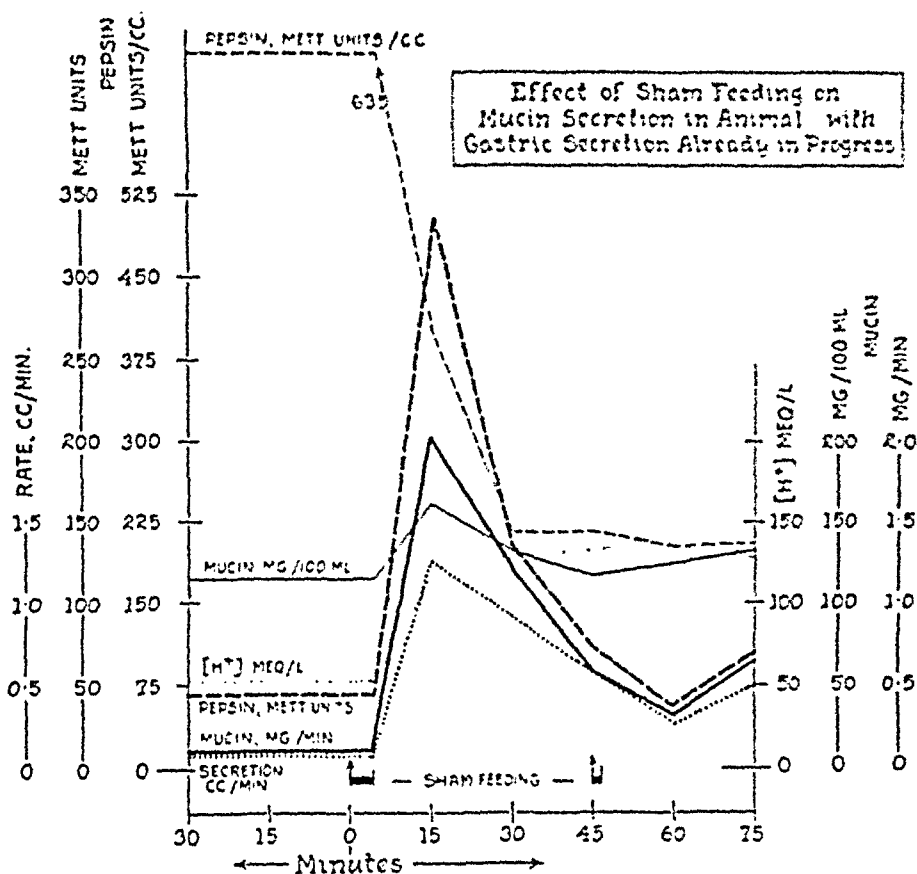


Fig. 2

## DISCUSSION

It has been demonstrated in this investigation that the output of so-called 'dissolved mucin' is greatly stimulated by sham-feeding or 'conditioned stimuli' associated with it, while histamine administration does not influence mucin secretion and under certain conditions appears to depress it. Qualitatively, the secretory response of mucin and pepsin was similar with both kinds of stimulation, but quantitatively there was a lack of parallelism in the concentrations of these two major constituents of gastric juice. Even more marked was the absence of any parallelism between mucin concentration and acidity. These findings suggest the existence of some essential differences in the mechanisms governing the function of the respective secre-

tory cells, both in response to sham-feeding and to histamine. At the present time we can make no definite statements concerning the nature of these differences.

Two questions naturally arise regarding the increase in output of mucin after sham-feeding: First, could the increase in secretion of mucin be a secondary effect, the result of an increase in gastric motility which might be induced by sham-feeding? This may be answered in the negative, since the results of other studies (7) show that sham-feeding inhibits gastric motor activity already present and that the output of mucin is greatly increased (3 to 5-fold) during the periods of motor inhibition (8) following the sham-feeding. Second, could the increased output of mucin after sham-feeding be a secondary effect of local stimulation by the acid of the secreted juice? That this is not so is indicated by the results of our experiments with histamine, since in these experiments the output of mucin was much lower than it was in the sham-feeding experiments in spite of comparable rates of secretion and equal or even higher acidities. Thus, the increased output of mucin after sham-feeding and after

TABLE 2. SECRETION OF MUCIN: RESPONSE TO HISTAMINE IN ANESTHETIZED DOG

SPEC.	RATE	[H +]	TOTAL ACIDITY	CHLORIDE	PEPSIN METT UNITS		MUCIN		P/M
no.	cc/min.	mEq/l.	mEq/l.	mEq/l.	/cc.	/min.	mg/100	mg/min.	ratio
1	0.24	102	136	149	7.8	1.83	53.3	.128	.146
2	0.72	117	146	162	0.6	0.43	17.2	.124	.035
3	0.89	120	148	164	<0.1	<0.1	4.9	.044	<.020
4	0.97	123	148	156	<0.1	<0.1	4.9	.047	<.020
5	0.83	126	149	159	<0.1	<0.1	5.6	.046	<.018

Acute experiment. Dog ♀ 8.3 kg. Pentobarbital sodium anesthesia. Gastric fistula. Pylorus and esophagus (in the neck) ligated. Histamine dihydrochloride injected for one hour before collection of sample 1 (0.2 mg. at 15-minute intervals) and during collection of samples 1, 2 and 3. The loss of body fluid was compensated by intravenous injections of saline at 15-minute intervals.

P/M = Ratio of pepsin concentration to mucin concentration.

stimulation with related 'conditioned stimuli' should be regarded as the direct result of reflex stimulation of the mucin secreting cells.

The fact that the decrease in the concentration of mucin in response to histamine was not as pronounced as that of pepsin may be the result of a local stimulatory action of the acid of the juice on the mucin secretion, although convincing evidence in favor of this hypothesis is not available at the present time.

#### SUMMARY

Experiments performed on dogs with gastric fistula and esophagotomy demonstrated conclusively that sham-feeding causes a pronounced increase in the output of 'dissolved mucin' as effectively as it does that of acid and pepsin. Furthermore, an analysis of the curves for concentration and output of mucin, pepsin and acid shows the response of each to be independent, an indication that the respective sets of secretory cells respond in a selective manner to sham-feeding. Similar effects were obtained also with 'conditioned stimuli' related to the act of sham-feeding. The stimulatory effect of sham-feeding was shown to be a primary one, independent of

changes in gastric motility, and not due to the local action of the acid of the gastric juice.

Experiments with histamine on atropinized, unanesthetized dogs and in sacrifice experiments under sodium pentobarbital anesthesia showed that the parietal cells alone are stimulated by this substance. The output of mucin remained constant at the level of the resting stomach secretion and with larger doses even appears to be depressed.

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# A COMPARISON OF THE PROPULSIVE MOTILITY OF THE SMALL INTESTINE IN ADULT DOGS AND PUPS

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**A**LTHOUGH the effect of ageing on the physiology of the gastrointestinal tract is a pertinent one, relatively little is known about this subject. This is particularly true of the motility of the tract. Carlson (1), as a result of his studies on hunger, concluded that in healthy dogs the hunger contractions of the empty stomach decrease with age. This decrease, however, was mainly in duration of activity. Studies of digestive motility in this laboratory (2) have revealed no difference in the gastric emptying time of aged men in comparison with that of a large group of young adults.

Some comparison between the progress of test meals through the entire digestive tract in infants and in adults is available in the literature. Lesné, Binet and Paulin (3), who used carmine to mark the ingested food in infants, found it appearing in the feces within 8 to 9 hours. Mulinos, in medical students also with carmine (4), found a great variability in the time of its appearance, but the greatest number of subjects passed it after 24 hours. Barium sulfate has been reported to appear in the feces within 24 hours in adult man (5), but as Alvarez has pointed out (6), longer intervals have been found, particularly with other test agents, by several investigators. If the living length of the entire intestine of infants bears the same relation to the living length of the adult intestine as that seen in cadavers (7), that is, about 40 per cent, and the rate of propulsive motility is the same in both, then, on the basis of results with infants one would expect a test meal to appear in the feces at around 24 hours in the adult. Since, so far as the available data indicate, this is about the time of appearance in at least some adults, it would seem that the propulsive motility may not necessarily depend on age.

Inasmuch as this laboratory had accumulated, in connection with other experiments, a large amount of data on the propulsive motility of the small intestine of adult dogs, it was deemed profitable to extend such investigations to pups in order to assess the age factor.

## METHODS

The experimental animals consisted of 111 canine pups and 126 adult dogs. The exact age of the pups, in most instances, was not known, so that the presence or absence of puppy teeth was used as a criterion for classifying the animals. In no cases were borderline (that is, those possessing both puppy and adult teeth) animals used. The body weight of the pups ranged from 0.355 to 5.50 kg. while the average was 2.03 kg. That of the dogs ranged from 2.49 to 19.2 kg. with the average at 7.59 kg.

Motility of the small intestine was determined essentially by Macht's technique for the following intervals of time: 5, 8, 15 and 30 minutes. Fifteen to 50 cc. (de-

pending on the size of the animal) of a mixture of 10 per cent powdered charcoal suspension in 10 per cent gum acacia in water was given by stomach tube to the animals, unanesthetized and in the post-absorptive state. At the end of the appropriate time intervals after intubation the animals were given a fatal dose of ether, the small intestine removed, slit open and the distance the charcoal mixture had traversed the intestine was measured.

### RESULTS

The length of intestine traversed by the charcoal mixture for each time interval for the two age groups is shown in figure 1, in which curves were visually fitted to the

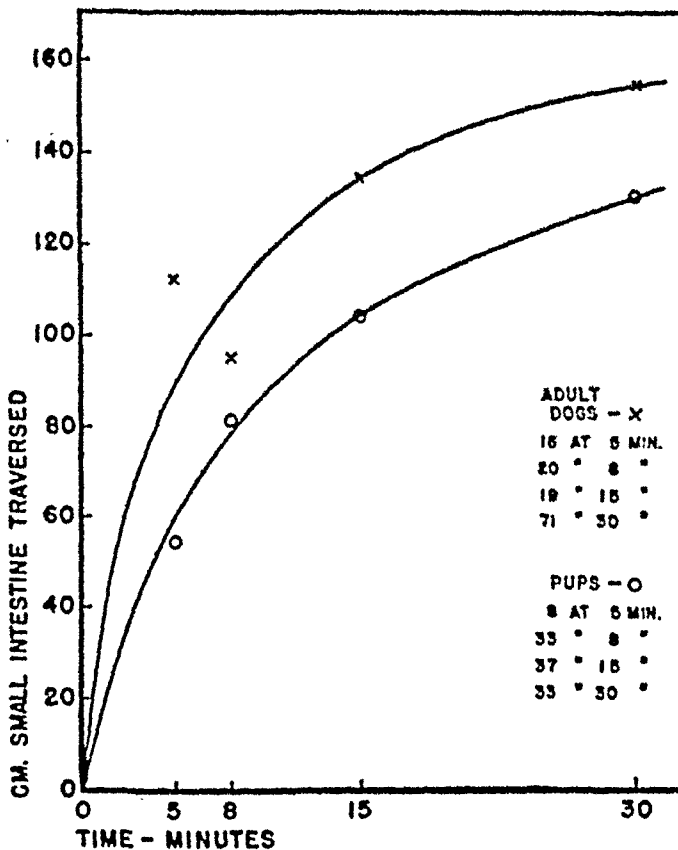


Fig. 1. LENGTH OF SMALL INTESTINE traversed at various intervals of time after gastric intubation with charcoal-acacia mixture.

experimental points. The fit is surprisingly good for the data, in which the variability is quite high. Since the curve for pups resembles that for dogs, similar factors are probably, though not necessarily, acting over time in both groups. However, the rate at which inert material is propelled in the small intestine is greater in dogs than in pups. This is true particularly at the end of 15 and of 30 minutes, where the differences in centimeters traversed were 31 and 24 with values of  $P$ : 0.026 and 0.022.

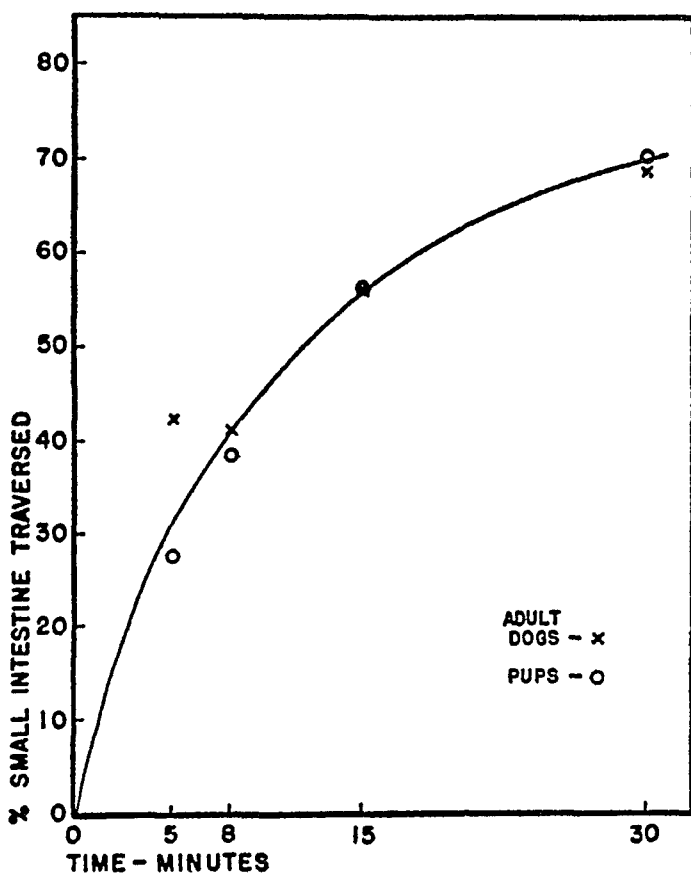
In figure 2 in which the data were plotted according to the percentage of the small intestine traversed in the two groups of animals, it is seen that the points fall essentially along one line. This suggests that, although rate of propulsion is slower in pups, the proportion of the gut traversed is similar for each time interval in pups and dogs.

The average length of the small intestine in the pups was 194 cm. and in the dogs, 233 cm. This turns out to be 96 and 31 cm/kg. of body weight respectively.

#### DISCUSSION

The method of measuring intestinal motility which has been used here does not yield absolute rates at which material is moved along the various parts of the small intestine. This is due to the fact that the stomach may not begin to empty promptly after intubation. For example, an occasional animal was found in which emptying had not occurred even within 30 minutes. The longer the interval over which the motility is measured the greater number of animals having delayed emptying would be ex-

Fig. 2. PERCENTAGE OF SMALL INTESTINE traversed at various intervals of time after gastric intubation with charcoal-acacia mixture.



pected to be included. Hence, delayed gastric emptying would theoretically introduce a factor which alone would tend to magnify falsely the true gradient of motility along the small intestine.

If delayed gastric emptying was equally present in both series of animals, a comparison of the results should be valid. If, for the sake of argument, the gastric emptying of pups had been more prompt, as might be inferred from Carlson's experiments on hunger contractions, the length of small intestine traversed would have been apparently greater when compared with that of adult dogs. Since, in spite of this possibility, the adult dogs had a fairly significant greater apparent motility, it seems inescapable that the true rate of propulsion must be greater in the small intestine of adult dogs.

An earlier determined (8) degree of correlation between length of small intestine and centimeters traversed after 30 minutes was so low ( $-+0.38$ ) that no great dependence of motility on gut length was visualized. In the present series of 33 pups which were killed at the end of 30 minutes, the degree of correlation is considerably higher:  $+0.59$ . This finding along with the overlapping curves for percentage of the small intestine traversed over time (fig. 2) in the two series make it quite probable that the rate of propulsion is related to the gut length. The faster rate seems to be found in the longer intestine and the slower rate in the shorter intestine.

It could be argued that in the best interests of the economy of the organism, the chyme should remain within the limits of the small intestine until the processes of digestion and absorption are complete. The time necessary for such completion would be expected to be fairly constant from individual to individual regardless of gut length. If some provision were not made for correlating rate of propulsion of chyme with the length of the viscus, there would be the possibility of incomplete digestion and absorption within the small intestine and the loading of the colon with food residues which might lead not only to wastage of food but also to improper functioning of the colon. Our results suggest, at least, that some sort of regulation may exist which prevents this possibility. It is of interest to speculate upon the nature of this control. The regulating mechanism may simply be inherent in the growth of the digestive tube and/or its nervous supply. On the other hand it is possible that some negative feed-back mechanism may control the progress of the chyme. Inasmuch as water was the main physiologically active component of the test meal used, the first alternative seems more probable; otherwise, the presence of water must be considered capable of influencing the motility of the intestine.

#### SUMMARY

The length of small intestine traversed by a charcoal-acacia suspension in 5, 8, 15 and 30 min. has been determined in 126 adult dogs and in 111 pups. The rate of propulsion was found to be greater in adult dogs than in pups, but the proportion of the small intestine traversed per interval of time was found to be essentially the same in both. The latter result along with the finding of positive correlations between gut length and rate of propulsion suggest that the speed of propulsive motility may be related to gut length. The physiologic significance of such a relation is discussed.

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# EFFECT OF INTRAVENOUS CALCIUM SALTS ON RENAL EXCRETION IN THE DOG<sup>1</sup>

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**F**EW systematic studies of renal excretion following intravenous administration of calcium salts appear in the literature. This report of 25 experiments on 7 dogs (15.6–25.5 kg.) concerns some urinary functions of the kidney which became evident during the course of steady intravenous injections of solutions of calcium chloride or calcium gluconate.

## PROCEDURES

A wing tip, soft rubber catheter was introduced into the bladder of a perineotomized female dog. The animal was allowed to stand in a stall in which the only restraint consisted of a loose strap or loop of cloth around each leg, held high on the leg by a supporting chain. No restriction to movement was applied unless the dog attempted to walk away or lie down. The former attempt was uncommon once the dog perceived the nature of the restraint and the latter occurred with some frequency only in certain dogs which appeared to rest comfortably on the straps for long periods. Urine was readily collected at hour or half-hour intervals by unstopping the distal end of the catheter hanging between the hind legs, with or without the necessity of routine manual pressure on the abdomen in the region of the bladder. Analyses were performed on 6 hourly urine specimens to determine  $pH$ ,  $HCO_3$ ,  $Cl$ ,  $Na$ ,  $K$ ,  $Ca$ ,  $Mg$ , inorganic  $PO_4$ , and inorganic  $SO_4$ . Serum was obtained during a control hour before starting intravenous infusion and during the 5th hour of infusion, and was analysed for the same substances as the urine except that  $SO_4$  and  $pH$  were not determined. Infusion of solutions of calcium chloride or calcium gluconate of specified concentration was carried out either at a 'fast' rate (averaging  $4.18 \pm 0.22$  cc/min.) or at a 'slow' rate (averaging  $0.586 \pm 0.088$  cc/min.) by means of a pump. The solution was forced through an 8-inch length of pure polyethylene plastic tubing, about 3 inches of which were introduced into the lateral superficial vein of a lower hind leg through a gauge 15 needle. The latter was withdrawn and the plastic tubing taped in place. Fluid entered the plastic tubing through a gauge 25 needle connected by rubber tubing to the infusion pump. All experiments were carried out in a room whose air temperature was held between  $21^\circ$  and  $23^\circ$  C.

## METHODS

Inorganic  $SO_4$  was determined by the method of Fiske (1) and inorganic  $PO_4$  by that of Fiske and Subbarow (2). Magnesium was analysed colorimetrically (3) and  $Ca$  titrimetrically (4). Other

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analyses were carried out as indicated previously (5). Because of the number and complexity of combinations of data obtainable from even a few experiments in which so many variables are studied simultaneously it seemed necessary to group the data in some ways arbitrarily and to test the statistical significance of many of the results by the 't' test (6).

Infused solutions will be considered to belong to one of 3 groups according to the rate at which calcium was infused. *Group A*, or zero calcium, includes only infusions of 5½ per cent glucose; *group B*, or medium calcium, includes infusions of calcium ranging from 7.76 to 37.5 micro-equivalents per minute<sup>2</sup>; and *group C*, or high calcium, includes infusions ranging from 59.2 to 117 micro-equivalents per minute. Higher rates of calcium administration could not be tolerated for 5 hours without the intervention of vomiting and such rates of calcium administration could induce vomiting even before appreciable loads of calcium developed.

### RESULTS

Figure 1 shows the relation between the actual rate of urinary flow ( $u$ ) and the duration of infusion for the three infusion groups when these were given both at 'fast' and 'slow' rates of fluid input. No specific diuretic or eucletic effects of calcium appear to exist independently of or in conjunction with those of water or isotonic glucose, i.e., urinary flow in non-hydrated (average net load of water at the end of the 5th hour = -28.7 cc.) and in hydrated (average net load of water at the end of the 5th hour = 267 cc.) dogs shows no influence traceable to differences in the simultaneous Ca load<sup>3</sup>.

Figure 2 indicates how average urinary  $pH$  values varied with the duration of the infusion. Whereas little importance need be attached to the  $pH$  values at the beginning of the infusions, those values by the 5th hour are suggestive. At the 5th hour the zero (*A*), medium (*B*), and high (*C*) calcium groups form a series of increasing urinary acidities as we go from *A* to *C*, regardless of the initial level of the urinary  $pH$ . From the data at hand it could be shown that *groups A* and *B* combined differed significantly from the *C* groups. Gluconate and chloride were not different in their effects on urinary  $pH$ . In any case, these are not 'acidifying' effects of the kind seen with oral calcium chloride. No significant change of serum  $HCO_3$  was demonstrated following calcium infusion. Urinary  $HCO_3$  excretion, in accord with  $pH$ , showed significantly lower values in *C* groups than in *A* and *B* groups combined at the 5th hour.

Figure 3 illustrates relations between the excretion rates of both Cl and Ca and the corresponding infusion rates of these ions. The diagonal passing through the origin is the locus of all points at which excretion rate equals intake rate ( $uU = iI$ ). It is seen that with intakes below approximately 2 micro-equivalents per minute, renal excretion of Ca is greater than intake so that the body develops an increasingly negative load of Ca as the duration of infusion is prolonged. For Cl, this *minimal isorrheic quantity* (MIQ) is of the order of 8 micro-equivalents per minute. Below this rate of intake, Cl escapes from the body faster than it is put in; above this rate and below approximately 70 micro-equivalents per minute its load in the body increases in the course of an infusion of  $CaCl_2$ . At intake rates in excess of 70 micro-

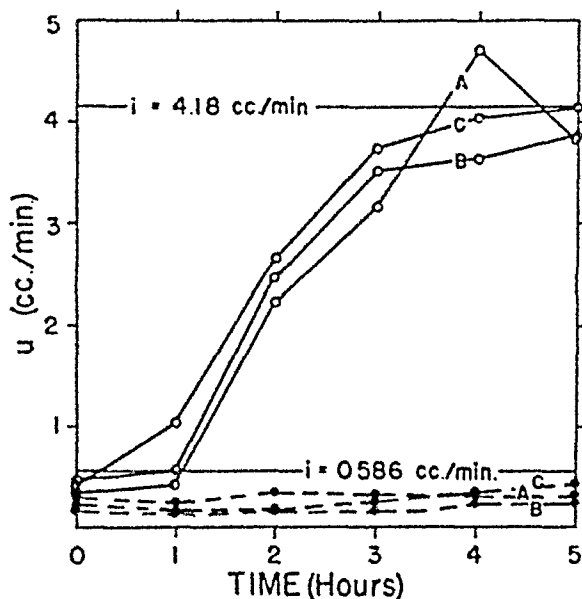
<sup>2</sup> Dilute solutions were fortified with sufficient glucose to make them isotonic.

<sup>3</sup> Insensible water loss,  $w$ , was estimated to be 0.4 cc/min. (7) or 120 cc. for the duration of the infusion. It may be observed in figure 1 that  $u + w > i$ , in accord with the expected euresis of plain water intake<sub>9</sub>, (10).

equivalents per minute the rate of excretion of Cl again exceeds its rate of intake and negative loads of Cl develop. Extending the nomenclature of isorrheic concentrations (7-10, 5) we find that this second point of equivalence between intake and output of Cl would be a *non-limiting isorrheic quantity* (NLIQ). A *limiting isorrheic quantity* (LIQ) for Cl would be difficult to demonstrate since the toxicity of higher infusion rates of Ca precludes ascertaining a point of intake of  $\text{CaCl}_2$  above which the urinary output of Cl is less than its intake at the 5th hour (see DISCUSSION). The MIQ and the LIQ for Ca appear to be identical. Figure 3 shows that Cl is excreted far more rapidly than Ca.

In figure 4, relating the excretion rate of Ca to the load of Ca at the 5th hour, the slope of the curves represents the velocity constant of excretion of Ca ( $\gamma = uU/L =$  rate of excretion per unit load where load is expressed in micro-equivalents and ex-

Fig. 1. URINE FLOW ( $u$ , in cc/min.) as it varied with the duration of infusion (time, hrs.). Solid lines connecting open circles represent urinary flow during 'fast' infusions, the average rate of which is represented by horizontal line  $i = 4.18$  cc/min. A (zero Ca) 4 cases; B (medium Ca), 2 cases; C (high Ca), 5 cases. Interrupted lines connecting black circles represent urinary flow during 'slow' infusions, the average rate of which is represented by the horizontal line  $i = 0.586$  cc/min. A, 3 cases; B, 7 cases, and C, 4 cases.



cretion rate in micro-equivalents per minute). Calcium excretion rates were not shown to be significantly higher with 'fast' rates of fluid intake (or, therefore, with greater urinary flow) than with 'slow' rates. In figures 5 and 6 the rates of excretion of different ions are expressed as a percentage of the pre-infusion rate of excretion (100%) of the control hour. Pertinent information from these graphs may be summarized as follows:

1) Ca excretion increases as Ca intake (and Ca load) increases, with no significant differences attributable to the anion.

2) During infusion, Cl excretion increases with increase of Cl intake; not with increase of water or gluconate (or, therefore, of Ca).

3) Mg excretion appears to increase as the load of Ca increases and to decrease as the load of water increases. Where water alone is given rapidly Mg output decreases and where Ca is given with little water, Mg output increases. The two factors oppose each other to the extent that where both Ca and water are given rapidly ('fast' C), no significant change in Mg excretion occurs.

4) Na excretion is specifically increased by high Ca intake, by the 5th hour. It is decreased by infusions containing no Ca.

5) K excretion is not specifically decreased by Ca intake; it falls significantly during infusions of solutions with medium or zero Ca. However, high Ca tends to oppose in latter periods the decreased K excretion which might otherwise occur.<sup>4</sup>

6)  $\text{PO}_4$  output is significantly decreased both by increased water intake and by increased Ca intake. The effects are not synergistic.

7)  $\text{SO}_4$  excretion is not significantly affected by either water or Ca intake.

Table 3 presents some computations which should yield the volume of distribution of Ca if the conditions set forth by Dominguez (11) and Crandall and Anderson (12) obtained. Using large Ca loads and large plasma Ca increments, a volume of distribution for Ca of the order of 65 per cent of the body weight is calculated. The meaning of this result is not clear since with lower or negative Ca loads calculations did not agree with those at high loads. Possibly the presence of high Ca in body fluids is conducive to singular results. A volume of distribution obtained by means of radioactive Ca would conceivably reveal still other volumes of distribution by computation.

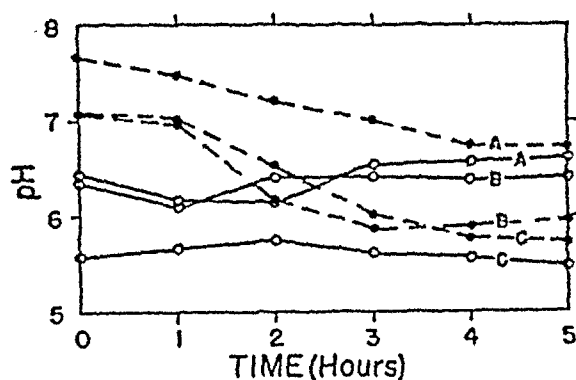


Fig. 2. RELATION OF URINARY pH to duration of infusion (time, hours). Solid lines represent 'fast' infusions; interrupted lines represent 'slow' infusions. The pH values of groups A and B combined differ significantly from groups C. See text for details.

## DISCUSSION

*Calcium and Water Excretion.* Definitive relations between renal excretion of water and calcium have never been drawn. Boekelman and Staal (13) perceived no relation in man between urinary flows and the excretion of Ca or  $\text{PO}_4$ . Mendel and Benedict (8) reported some diuresis following intravenous injections of  $\text{CaCl}_2$  in dog and rabbit but no clear indication of a specific diuretic effect of Ca was given. Goffart and Brull (14) observed increased Ca excretion with water diuresis in dogs and our own results suggest such an effect (fig. 4) although it was not proved statistically significant. Increased water intake, water load, and/or water excretion may augment the excretion of Ca but it appears from our data that increased Ca intake, load, and/or Ca excretion do not effect increased urinary output of water. One purpose in carrying out Ca infusions at two different rates of water intake (the lower intake approximately set to maintain the dog in water balance throughout the experiment) was to elucidate any specific diuretic effect of Ca which might exist independently of water load; none was found. However, instances where water load influences the action of given

<sup>4</sup> The 'specificity' of Ca effects may be only relative. It is not shown that the dekalifying effect of 'slow' C infusions is not primarily or in part a consequence of the induced hypertonicity of body fluids (5).

diuretics abound, e.g., drugs of the caffein group act diuretically in the dog when there is excess water present, and antiduretically in water deficit (15); mercurial diuretics are more effective in the presence of edematous water loads (16); and 'osmotic' diuretics lose specificity of action during water diuresis (17).

*Thresholds.* Albright and Ellsworth (18) studied a case of idiopathic hypoparathyroidism before and after the administration of parathormone. They believed that

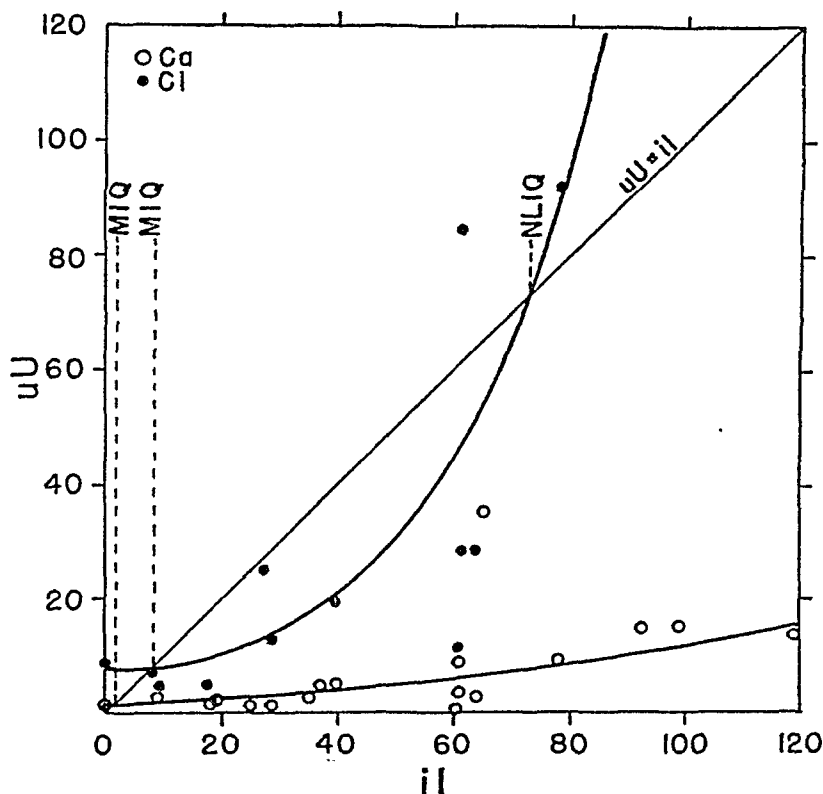


Fig. 3. RELATION BETWEEN RATES OF EXCRETION of Ca and Cl (uU, in micro-Eq/min.) and rates of intake of Ca (iI, in micro Eq/min.), at the 5th hour of infusion ('slow' and 'fast' series combined). Cl excretion (black circles) is plotted only for infusions of  $\text{CaCl}_2$ ; Ca excretion (open circles) is plotted for infusions of both  $\text{CaCl}_2$  and Ca gluconate. The diagonal passing through the origin represents equality of rates of excretion and intake ( $uU = iI$ ). See text for significance of *minimal isorrheic quantity* (MIQ) and *non-limiting isorrheic quantity* (NLIQ). The Cl curve is a good fit through all points, including 2 not shown on graph at high uU and high iI values (see table 1). The Cl and Ca points at  $iI = 0$  are averages of 7 values.

there was a threshold of excretion (appearance) for Ca at about 4.25 mEq/l. since at serum levels above this, the urinary excretion of Ca suddenly increased. Their data indicate that urinary concentration of Ca generally exceeded the plasma concentration of Ca when the latter was above normal values (i.e., approximately 5 mEq/l.), which would further suggest the existence of a threshold of retention (9, 5) for Ca at about the normal plasma level. Parathyroid hormone has been supposed to exert a direct effect on Ca and  $\text{PO}_4$  excretion (19, 20-22) but this is considered less a renal effect than an extra-renal one since it depends on the action of the hormone in mobilizing Ca from body stores rather than on specific effects on tubular reabsorption of Ca or  $\text{PO}_4$ . Brull (23) claims a direct action of parathormone in

TABLE I

SOLUTION	GROUP AND EX- PER. NO.	DOG	i	H <sub>Ca</sub>	TIME	u	u <sub>Ca</sub>	u <sub>Cl</sub>	u <sub>Na</sub>	u <sub>K</sub>	u <sub>ug</sub>	u <sub>HCO<sub>3</sub></sub>	u <sub>SO<sub>4</sub></sub>	u <sub>FO<sub>4</sub></sub>	URIN- ABSORP	I <sub>Na</sub>	I <sub>Ca</sub>
5½% Glucose	A-23	Q	0.800	0	0	0.393	0.07	16.9	31.0	14.2	1.10	18.8	15.7	0.64	8.02	-69	-20.8
5½% Glucose	A-29	M	0.564	0	5	0.700	0.04	6.30	3.50	2.73	1.33	1.43	7.70	3.80	6.63		
5½% Glucose	A-30	N	0.347	0	0	0.152	0.33	9.73	14.4	19.5	0.49	5.84	15.8	2.60	8.26	3	-119
5½% Glucose	A-34	K	0.596	0	5	0.166	0.50	5.98	9.30	3.66	0.93		6.65	5.13	7.69		
					0	0.591	0.30	37.8	39.0	13.6	3.61	1.05	17.7	14.3	6.42	-134	-64.3
					5	0.367	0.18	6.90	5.50	4.80	1.87	0.76	5.90	5.20	6.73		
					0	0.128	0.21	4.22	20.0	5.84	0.81	0.41	10.8	0.94	8.00		
					5	0.100	0.21	0.60	1.20	3.90	0.15		30.0	0.22	6.30	27	-59.2
CaCl <sub>2</sub>	B-33	N	0.473	27.0	0	0.219	0.28	16.2	16.6	19.3	1.88	0.50	21.9	26.4	6.48		
					5	0.367	1.21	12.1	1.84	1.80	1.94	0.50	14.7	0.11	6.40	-34	7000
					0	0.176	0.24	28.8	33.2	16.9	2.04	11.2		5.57	7.63		
Ca gluconate	B-25	K	0.604	37.1	5	0.150	4.85	10.7	15.5		2.04			0.68	5.40	46	10500
					0	0.491	0.84	65.3	49.1	10.8	4.71	0.14	18.7	15.4	5.57		
CaCl <sub>2</sub>	C-22	Q	0.857	59.2	5	1.18	8.56	84.0	39.0	6.15	4.96	0.26	11.8	0.95	5.40	-55	16000
					0	0.167	0.25	25.0	19.5	39.0	1.00		16.0	7.11	7.47		
CaCl <sub>2</sub>	C-26	D	0.576	61.6	5	0.115	0.79	28.3	1.38	5.75	5.10		10.3	0.41	6.28	28	18400
					0	0.147	0.18	3.53	7.05	8.46	0.53		15.0	1.07	6.47		
CaCl <sub>2</sub>	C-27	K	0.533	59.6	5	0.117	3.63	11.2	2.10	1.54	1.69		10.5	0.27	6.37	8	17200
					0	0.237	0.38	21.3	20.4	45.0	2.23	5.09	9.48	15.8	7.51		
CaCl <sub>2</sub>	C-35	E	0.457	80.5	5	0.267	9.35	92.4	42.5	13.4	6.84	0.69	21.4	1.68	5.60	-51	22600
					0	0.250	0.18	3.50	13.0	10.0	1.05	13.4	0.60	0.20	8.30		
CaCl <sub>2</sub>	C-28	Q	0.550	117	5	0.666	13.5	238.	130	30.6	11.3	0.01	26.6	0.13	5.02	-65	33100
					0	0.37	0.56	61.5	70.4	77.0	7.21	36.2	33.3	22.8	7.58		
5½% Glucose	A-7	K	4.00	0	5	4.01	7.23	8.00	16.0	6.43	0.80	7.10	20.1	0.16	6.50	149	-1450
5½% Glucose	A-21	N	4.30	0	5	0.131	0.20	12.4	14.8	7.20	1.62		13.6	9.23	6.23		
					0	4.26	0	4.26	8.51	8.95	0.51	15.0	9.36	4.26	6.82	257	-386
5½% Glucose	A-16	N	3.90	0	5	0.534	4.10	154	49.6	85.5	14.4	0.12	23.5	15.7	5.51		
					5	3.19	1.76	25.6	6.39	5.75	0.86	6.29	16.3	1.69	6.50	329	-613

CaCl <sub>2</sub>	B-15	P	3.88	7.76	0	0.623		29.3	16.8	11.7	2.05	2.69	5.60	1.75	6.82		272
CaCl <sub>2</sub>	B-17	M	3.93	8.25	5	3.83	7.66	7.66	3.83	3.83	3.26	7.78	25.3	0.08	6.61		
Ca gluconate	B-12	K	4.26	19.0	5	0.138	0.26	15.9	20.8	19.0	3.92	4.50	32.0	10.1	6.87		2080
CaCl <sub>2</sub>	B-20	K	4.24	18.9	5	4.13	2.48	4.13	8.25	3.72	2.97	4.50	20.6	0.02	6.39		
CaCl <sub>2</sub>	B-18	Q	4.20	21.0	5	0.183	0.38	47.0	11.3	31.3	2.49	0.01	14.6	6.07	5.62		5260
Ca gluconate	B-9	N	4.22	35.7	5	4.06	2.03	8.14	8.14	8.14	0.81	4.37	0	0.81	6.21		
CaCl <sub>2</sub>	B-11	D	4.34	37.5	5	0.117	0.36	14.0	10.2	16.3	2.27	8.42	8.42	0.86	7.34		
Ca gluconate	C-13	N	4.00	65.2	5	4.44	1.33	4.44	35.4	4.44	0.04	6.43	20.8	0.35	6.40		5210
CaCl <sub>2</sub>	C-14	E	4.07	67.0	5	0.625	1.66	54.4	4.37	22.5	6.00	0.14	12.5	10.1	5.75		
Ca gluconate	C-10	M	5.24	99.0	5	3.06	1.07	27.6	12.3	3.68	10.4	4.16	12.2	0.18	6.49		5900
CaCl <sub>2</sub>	C-19	Q	3.92	93.0	5	1.39	4.86	144	8.35	89.0	4.57	0.25	26.4	24.8	5.33		
					5	4.25	2.55	10.2	4.25	5.10	1.27	4.04	8.50	0.17	6.22		9790
					5	0.263	0.58	605	20.5	52.4	0.76	3.08	5.25	14.6	6.78		
					5	3.06	5.20	19.6	15.3	3.98	0.15	5.20	18.4	0.26	6.47		10500
					5	0.673	3.69	98.0	11.4	34.2	3.22	0.19	6.73	25.9	5.51		
					5	4.40	12.8	35.2	8.80	11.9	1.84	2.24	14.4	0.40	5.82		1690
					5	0.460	0.60	70.4	29.0	64.9	1.88	0.07	25.3	22.2	5.39		
					5	4.00	2.80	28.0	8.00	3.60	0.60	6.36	14.4	9.25	6.39		19800
					5	0.218	0.67	45.1	3.27	31.4	15.2	0.02	16.1	17.4	5.44		
					5	4.09	17.2	38.4	81.6	26.9	1.02	0.20	16.3	0.20	4.90		27000
					5	0.392	0.41	22.0	22.4	9.8	4.55	0.24	18.0	10.2	6.03		
					5	4.14	17.2	211	149	33.1	10.8	0.25	4.14	0.41	4.87		24700

Control and 5th hour (time - 0, 5) data for solutions of CaCl<sub>2</sub> and Ca gluconate. Groups A, B, and C represent zero, medium, and high rates of infusion for calcium (iCa<sub>0</sub>), respectively, expressed in micro-Eq/min. Other symbols are: i = rate of intake of infused water in cc/min.; I = concentration of solute in infusion fluid in mEq/l.; iCa = rate of intake of Ca in micro-Eq/min.; u = urinary flow in cc/min.; U = urinary concentration of solutes (indicated in subscript) in mEq/l.; uU = urinary excretion of solute (indicated in subscript) in micro-Eq/min.; L<sub>H<sub>2</sub>O</sub> = net load of water in cc. at the end of the 5th hour, i. e., total water infused - (total water excreted in urine + 120 cc. of estimated insensible water loss); L<sub>Ca</sub> = load of Ca in micro-Eq. at end of 5th hour, i. e., total Ca infused - total Ca excreted in urine. Upper series of experiments are 'slow', lower series, 'fast.'

lowering the threshold of  $\text{PO}_4$  in the dog since the hormone was found to increase the excretion of  $\text{PO}_4$  without raising the plasma concentration of  $\text{PO}_4$ . Our own data are consistent with the idea that Ca loads raise the threshold of  $\text{PO}_4$  since they induce decreased  $\text{PO}_4$  excretion along with an increase (if any change) in plasma  $\text{PO}_4$  level. Whether the formation of an unfilterable colloidal complex of calcium phosphate (24) is at the basis of this effect is not established.

TABLE 2

EXPER. NO.	$\text{H}_{\text{Ca}}$	Ca		Cl		Na		K		Mg		$\text{HCO}_3$		$\text{PO}_4$	
		$A_0$	$A_1$	$A_0$	$A_1$	$A_0$	$A_1$	$A_0$	$A_1$	$A_0$	$A_1$	$A_0$	$A_1$	$A_0$	$A_1$
A-23	0	5.6	4.9	109	110	153	150	5.1	4.7	1.4	1.2	23.6	23.2	2.7	3.3
A-29	0	5.5	5.4	124	121	154	153	4.8	4.7	1.2	1.4	24.3	25.3	2.1	2.6
A-30	0	6.4	6.0	103	100	147	144	4.4	4.7	1.2	1.4	23.5	23.7	3.3	2.9
A-34	0	5.6	5.4	112	106	146	148	4.4	4.3	1.8	1.6	22.6	22.1	2.4	2.4
B-33	27.0	5.8	6.3	112	109	143	140	4.8	4.7	1.4	1.6	23.6		2.9	3.6
B-25	37.1	5.9	6.5	104	100	137	130	4.7	4.0	1.5	1.5	22.8	23.3	2.3	2.9
C-22	59.2	5.3	6.4	110	112	142	138	4.4	4.0	1.6	1.4	23.6	24.7	3.0	3.6
C-26	61.6	6.1	7.6	114	112	147	143	4.5	4.6	1.4	1.8	21.8	24.8	2.7	3.4
C-27	59.6	5.7	6.8	105	111					2.0	1.8	24.7	21.4	3.0	3.2
C-35	80.5	5.4	7.7	116	113	149	147	4.4	4.5	1.4	1.6	20.3	22.4	3.0	4.0
C-28	117	5.3	8.1	110	115	156	152	5.1	4.7	1.6	1.0	21.4	21.1	2.4	3.6
<hr/>															
A-7	0	6.3	6.4		116	153	148	4.0	4.0						
A-21	0	6.0	5.3	113	110	146	138	4.8	4.0	2.4	2.6	21.5	23.5	3.4	2.9
A-16	0	5.1	4.9	122	114	142	140	4.7	3.9			18.6	19.7	2.9	3.5
B-15	7.8			116	115	146	138	4.7	4.0			21.2	18.8	3.1	3.3
B-17	8.3	5.9	5.4	117	113	159	148	4.7	4.1			23.2	23.1	2.7	1.7
B-12	19.0	5.7	5.9	112	106	145	137	4.2	4.0			22.0	21.8	3.3	2.7
B-20	18.9	6.3	6.4	110	106	150	149	3.8	3.9	1.0	1.2	21.8			
B-18	21.0	5.3	5.6	113	107	147	144	4.4	4.1			20.4	21.8	2.7	2.7
B-9	35.7	5.3	7.2	113	110	146	150	5.8	5.4			17.0	20.4	3.0	3.1
B-11	37.5	6.4	8.2	113	110	159	150	5.3	4.5			25.8	23.3	2.4	2.6
C-13	65.2			112	106	149	145	4.7	4.0			19.3	22.3	2.9	3.3
C-14	67.0	6.1	7.2	112	114	143	130	4.8	4.3			20.5	19.6	3.5	3.5
C-10	99.0	4.9	8.2	111	94.6	150	140	4.9	3.7			23.4	23.0	2.3	2.4
C-19	93.0	7.3	9.0	116	128	155	148	4.4	4.3	1.8	1.2	24.2	22.9	3.3	3.7

Serum concentrations in mEq/l. in control period ( $A_0$ ) and in middle of 5th hour of infusion ( $A_5$ ). Other symbols as in table 1. Upper series of experiments are 'slow'; lower series, 'fast.'

While there may actually be a threshold of retention for Ca as suggested by the fact that concentration ratios ( $U/A$ ) either greater or less than 1 are seen, our data indicate that there is, effectively, no such threshold. The threshold of retention for a substance expresses that regulatory aspect of urinary function concerned with maintaining the normal plasma concentration of the substance. When water is loaded on the body ('fast' intake) simultaneously with Ca, the urinary concentration of Ca is below that of the infusion fluid when the latter is above or even somewhat below the concurrent serum concentration. This indicates that even under the moderate physiological stress provided by loads of relatively dilute solutions, there is no effective

renal regulation of plasma Ca, i.e., Ca is not excreted more rapidly than water, when that process is required if normal plasma concentration is to be restored. Unlike the case with Na or Cl when solutions of NaCl are given (5), there is no renal neutralization of the intake factors tending to alter plasma Ca. Presumably the bones and other tissues are the prime regulators of plasma Ca, acting in one sense as emunctories. Mendel and Benedict (8) and McCance and Widdowson (25) find little evidence that the gut removes parenteral Ca<sup>5</sup>.

*Isorrheic Concentrations and Isorrheic Quantities.* Na and Cl in the urine following steady infusions of NaCl solutions have been shown to possess critical isorrheic concentrations, i.e., concentrations of urine or infusion fluid at which there is no relative retention of water to ion or ion to water, where the velocity constants of

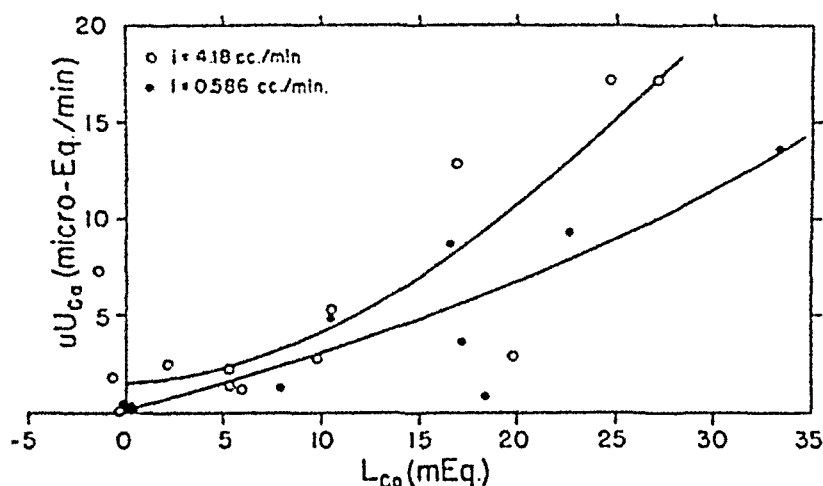


Fig. 4. RATE OF EXCRETION of Ca at the 5th hour ( $uU_{Ca}$ ) as a function of the Ca load ( $L_{Ca}$ ) at the end of the 5th hour. Experiments include  $CaCl_2$  and Ca gluconate. Although separate curves suggest some augmented Ca excretion at 'fast' rather than 'slow' water intake (and therefore higher rather than lower urinary output) at given Ca loads, no significant differences were demonstrated between the 'fast' and 'slow' groups of data. Where  $L_{Ca}$  (in mEq.) is multiplied by 1000 (i.e., expressed in micro-Eq.), the slope of the curves represents  $\gamma$ , the velocity constant of excretion of Ca (minute rate of excretion per unit load).

excretion of water and of the ions are equal, and where urinary concentration equals infusion fluid concentration (7, 9). Renal regulation maintains normal plasma concentrations of these substances and isorrheic concentrations are found whether or not equality of intake and output for these ions has been attained. Following intravenous administration of  $CaCl_2$  the kidney apparently does not regulate plasma concentrations of these administered ions in the sense of restoring them to normal levels. The minimal and limiting isorrheic concentrations for Ca, which may be identical (fig. 3), are values less than its normal plasma concentration. They are not critical concentrations but depend on the rate of urine flow. Figure 3 shows, however, a *minimal isorrheic quantity* (MIQ) for Ca at about 2 micro-equivalents per minute, represent-

<sup>5</sup> It was suggested, but not proved, by Wolf (5) that K had a threshold of retention in man since an occasional concentration ratio less than 1 was suspected. It has repeatedly been found in dogs with 'fast' infusions that U/A ratios less than 1 can be obtained (tables 1, 2) which would appear to establish a threshold of retention for K in the dog, although the extent to which this reflects renal regulation of K is not clear.

ing the lowest rate of Ca intake which may be maintained equal to the simultaneous rate of Ca excretion. Rates of Ca intake in excess of this value lead to accumulation of Ca in the body without limit. Cl shows a MIQ of approximately 8 micro-equivalents per minute and a *non-limiting isorrheic quantity* (NLIQ) of the order of 70 micro-equivalents per minute at which, again, the rates of intake and output of Cl are equal. Theoretically, regardless of the toxic manifestations which might set in, it should be

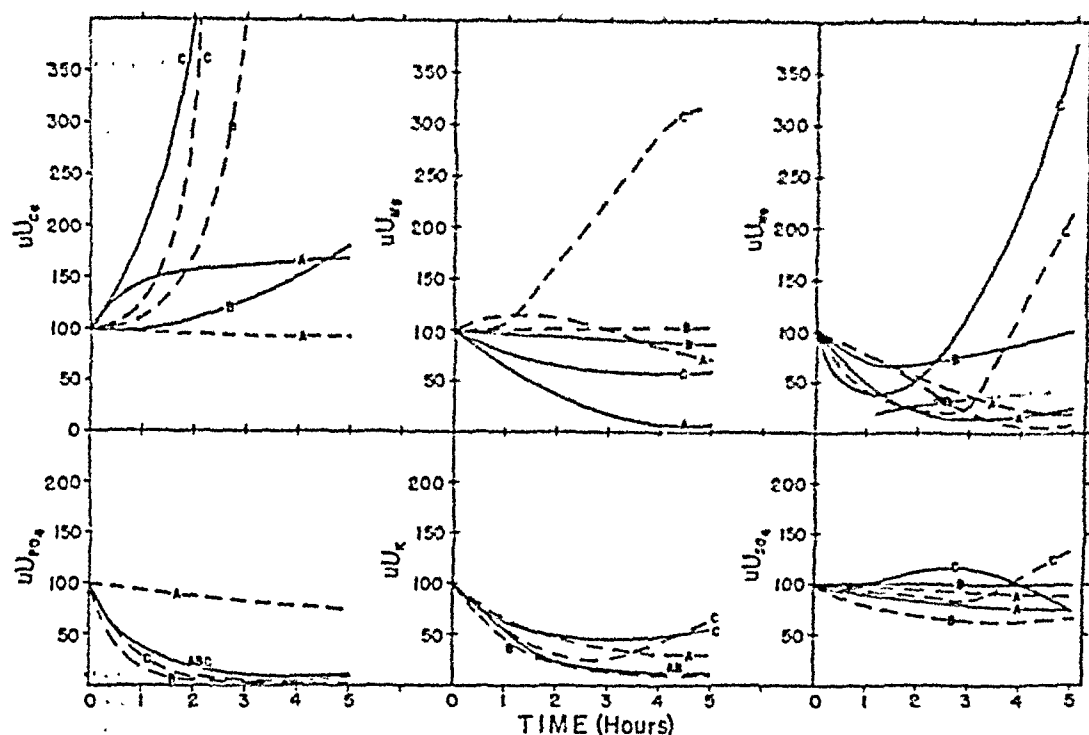


Fig. 5. RATES OF URINARY EXCRETION of Ca, Mg, Na,  $\text{PO}_4$ , K, and  $\text{SO}_4$  ( $u\text{U}_{\text{Ca}}$ ,  $u\text{U}_{\text{Mg}}$ , etc respectively) as a function of the duration of infusion. Average rate of excretion at zero time is taken as 100% for all groups. Solid lines represent 'fast' infusions (see fig. 1); interrupted lines represent 'slow' infusions. Significant differences in excretion rates were demonstrated only in the following cases: Ca, combined C groups from 0 to 5th hour; Mg, 'fast' A from 0 to combined 3rd, 4th, and 5th hours, and 'slow' C from 0 to 5th hour; Na, combined C groups from 2nd to 5th hour, and 'slow' A from 0 to 5th hour;  $\text{PO}_4$ , 'fast' combined A, B, and C from 0 to 5th hour, and 'slow' combined B and C from 0 to 5th hour; K, 'slow' A from 0 to 5th hour, 'slow' C from 3rd to 5th hour, 'fast' combined A and B from 0 to 5th hour, and 'slow' and 'fast' C combined from 0 to 2nd hour.

possible to complete a sigmoid form for the Cl curves so that a *limiting isorrheic quantity* (LIQ) could be shown, representing the maximal rate of intake of Cl above which the urinary output could not rise so high as the intake rate (i. e., the curve would again cross the diagonal  $uU = iI$  at a high  $iI$  value. See fig. 3)<sup>6</sup>.

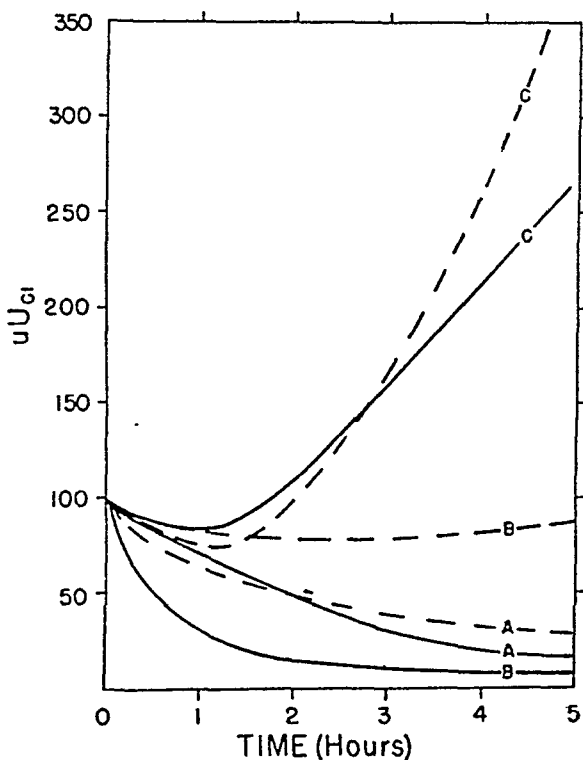
The relative excretion rate of Cl with respect to Ca when  $\text{CaCl}_2$  is infused is

<sup>6</sup> The highest urinary Ca concentration we have found in the literature is 53 mEq/l. reported for the dog by Mendel and Benedict (26) and observed following injection of  $\text{MgSO}_4$ . We discovered one urinary concentration of 75.7 mEq/l. in a dog which had vomited from an infusion of  $\text{CaCl}_2$ . The next highest values were only of the order of 35 mEq/l. The highest concentration ratio ( $U/A$ ) for Ca we have found has been 4.99. Concentration ratios close to zero are common when urine flow is high whether or not Ca is being administered. Normally the concentration ratio is greater than 1 in man (27).

given by  $\gamma_{Cl}/\gamma_{Ca}$  (5). This ratio was always greater than 1. Values greater than 100 are common;  $\gamma_{Ca}$  is of the same order (e.g., 0.0004 min.<sup>-1</sup>) or of lesser magnitude than  $\gamma_{Cl}$  when NaCl is given to dogs (7).

*Calcium and Other Electrolytes.* Among our results relating to altered renal excretion of electrolytes other than Ca, following Ca administration, the increased Mg excretion when water is not given in excess (fig. 5), is instructive. Mendel and Benedict (8) state that increased excretion of Mg accompanied increased excretion of Ca following priming doses of intravenous  $CaCl_2$  solutions. Some of the results shown in figure 5 confirm this. With regard to the apparently antagonistic action of positive water and Ca loads on the excretion of Mg, it is clear that the principle of 'washing

Fig. 6. RATE OF URINARY EXCRETION of chloride ( $uU_{Cl}$ ) as a function of the duration of infusion of  $CaCl_2$ . Average rate of excretion at zero time is taken as 100% for all groups. Solid lines represent 'fast' infusions (see fig. 1); interrupted lines represent 'slow' infusions. The combined C groups show statistically significant differences in excretion rates between 1st and 5th hours. Curves relating  $uU_{Cl}$  to time for Ca gluconate infusions (not shown) all closely resemble A and lower B curves, but significant decreases in Cl excretion were demonstrated for gluconate infusions for combined B and C groups from 0 to 4th hour.



out' of substances when urine flows are high does not apply. Although no significant changes in serum Mg were revealed in our experiments, it may be that the presence of a load of water in the body brings the plasma concentration of Mg below the threshold of retention so that Mg would tend to be excreted less readily. However, the specific effect of Ca on Mg excretion where water load is not increased remains unexplained.

Under the conditions of our experiments Ca promotes increased excretion of Na but not of Cl; Ca gluconate and  $CaCl_2$  both bring about the former but the gluconate does not increase the excretion of Cl.

An interesting phenomenon seen in figures 5 and 6 is the initial drop in excretion rate for certain substances (Cl, Na, K) preceding a later rise. Not all of these initial falls were demonstrably significant by statistical methods in these experiments but failure to demonstrate significance in a given number of trials does not militate

against the suggestion that the actual data characterize a fundamental phenomenon. Whether this effect is peculiar to the manner in which the present experiments were conducted or would be found under somewhat different conditions has not been decided. Results obtained under these conditions, except that the duration of the experiments be shorter, might lead an investigator to draw apparently antithetical conclusions. When loads of substances are added to the organism, the time during which they act or decay is not yet accounted for by current simplified theories of renal function based on initial renal responses.

Little systemic acidifying effect of intravenous  $\text{CaCl}_2$  was observed such as is seen following oral administration (28) nor was any systemic alkalinizing effect observed with Ca gluconate as reported by Lecoq (29). However, both types of Ca salt apparently favor the excretion of an acid urine (fig. 2). The mechanism of such acidification following intravenous  $\text{CaCl}_2$  of group C is probably different from that of oral administration since in the former case there is a loss of Cl and a retention of

TABLE 3

EXPER. NO.	$L_{\text{Ca}}$	$(A'_5 - A_5)_{\text{Ca}}$	b	B.W.	$\frac{b \cdot 100}{\text{B.W.}}$
22	16.6	1.33	12.5	19.0	65.7
26	18.3	1.62	11.3	25.5	44.5
27	17.2	1.19	14.5	15.8	91.5
35	22.6	2.55	8.87	17.2	51.6
28	33.4	3.14	10.6	19.0	56.0

Volumes of distribution, b, in liters, calculated from plasma Ca increments in mEq/l., by the end of the 5th hour  $(A'_5 - A_5)_{\text{Ca}}$ . Load of calcium,  $L_{\text{Ca}}$ , in mEq. The volume of distribution as a percentage of the body weight in kg. is given in the column headed  $b \cdot 100/\text{B.W.}$ .

Ca whereas in the latter case there may be a retention of Cl but little increment in body Ca.

#### SUMMARY AND CONCLUSIONS

Dogs receiving steady intravenous infusions of  $\text{CaCl}_2$  or Ca gluconate for 5 hours showed no specific diuretic responses traceable to the amount or rate of Ca administration.

Urinary pH tended to be lower following large doses of administered Ca than following small doses of Ca or when no Ca was infused. No significant effect of  $\text{CaCl}_2$  or Ca gluconate on alkaline reserve was found.

When  $\text{CaCl}_2$  was given intravenously, the velocity constant of excretion of Ca was much smaller than that of Cl and was of the same magnitude or less than the velocity constant of Cl when NaCl was given. Negative loads of Cl may be produced when  $\text{CaCl}_2$  is given.

Under the conditions of these experiments there is no effective threshold of retention for Ca, and plasma concentrations of this ion are apparently not regulated extensively by renal function. Relative retention of Ca to water occurs even when some solutions less concentrated than plasma Ca are administered. Minimal isorrheic

quantities for Ca and Cl were found to be 2 and 8 micro-equivalents per minute, respectively. A non-limiting isorrheic quantity for Cl was found at approximately 70 micro-equivalents per minute. Specific effects of Ca infusion on the excretion of other electrolytes were found, notably on Mg, Na, K, and PO<sub>4</sub>. The highest concentration ratio found for Ca was 4.99; the highest individual urinary concentration of Ca was 75.7 milliequivalents per liter.

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# EFFECT OF UREA DIURESIS ON RENAL EXCRETION OF ELECTROLYTES<sup>1</sup>

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**A**N ACCURATE description of the intrinsic renal mechanisms involved in the excretion of sodium has been difficult to obtain because of the errors inherent in the available experimental technics. Measurements reveal that less than one per cent of the filtered sodium is excreted by man in the normal state of fluid and electrolyte balance (1). Any attempt to define variations of sodium excretion in terms of changes in renal blood flow, glomerular filtration rate or tubular activity, would, of necessity, require analytical and experimental procedures with a combined error of less than one per cent. As such an accuracy is not readily obtainable in the study of the normal state, it is difficult if not impossible to distinguish between analytical errors and physiological variations in calculations designed to show changes in the intrinsic renal processes.

It was therefore thought that useful information might be obtained by studying electrolyte excretion during severe osmotic diuresis, utilizing the clearance technics at present available. Under such circumstances, the combined technical errors would be relatively small if large changes in water and electrolyte excretion were demonstrated, thus permitting a quantitative description of the interrelationships of the changes observed. Experiments were performed on dogs subjected to extreme diuresis by the intravenous administration of 50 per cent urea solutions. The results obtained seemed to justify the experimental procedure as they uniformly revealed phenomena interpreted as showing that sodium is reabsorbed by an active process in the proximal tubule, and that water back diffuses as a result of the osmotic gradient thereby established. In independent studies on mannitol diuresis in the dog, Wesson and Anslow (2) have come to a similar conclusion regarding the nature of the reabsorption of sodium. However, certain differences in the interpretations of the results of the two studies will be discussed.

## ANALYTICAL METHODS

Creatinine was determined by the method of Folin and Wu (3) with an Evelyn colorimeter. An internal standard flame photometer (4) with an accuracy of one per cent was used for the analysis of sodium and potassium. Urea was determined by the

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urease, aeration and titration method (5). Because of the high concentrations of urea no pre-aeration was used to correct for ammonia. Chloride was determined by a modified Volhard titration (6); inorganic phosphate by the method of Fiske and SubbaRow (7); and bicarbonate by the method of Van Slyke (8) on blood and urine collected under mineral oil.

#### EXPERIMENTAL PROCEDURE

Because of the nature of the experiments, dogs anesthetized with pentobarbital were used and, with the exception of two animals, were killed at the end of the experiment. The dogs were healthy male or female mongrels which had been fed on a high carbohydrate stock diet. Each experiment was preceded by an 18-hour fast during which the dogs were allowed free access to water. Urine was collected quantitatively over 20-minute periods by catheterization of female dogs and suprapubic cystotomy in males. Venous blood was taken in oiled syringes. Sodium oxalate was used as an anticoagulant for obtaining plasma for determinations of urea, creatinine and chloride. Blood samples were allowed to clot under oil and the serum was used for the determination of sodium and potassium. High concentrations of urea were rarely associated with slight hemolysis<sup>3</sup>, but this did not alter the determination significantly. Fluids were administered by infusion into the jugular vein.

Priming doses of creatinine (5 cc/kg.) in 5 per cent solutions were administered one half hour before observations were started. Plasma creatinine levels were maintained by continuous intravenous infusion. All animals were also primed with 2 per cent sodium chloride in water (30° cc/kg. body weight) and during the control periods infused with 1.5 per cent sodium chloride solution in quantities equal to urine flow. After the control periods, 50 per cent urea solution in saline was administered at a constant rate for each animal, varying from 1 to 4 cc. per minute. When the urea was administered the sustaining solution was changed to 0.9 per cent sodium chloride in water and urine volume was balanced by this infusion for the duration of the experiment. In 3 experiments low filtration rates were inadvertently obtained due to an untoward reaction to the priming dose of creatinine.

#### CALCULATIONS AND ABBREVIATIONS

Clearances were calculated from the mid-point plasma concentration and no correction was made for dead-space time because of the high rates of urine flow.

1.  $\left. \begin{matrix} (S)_{Na} \\ (S)_K \\ (S)_u \end{matrix} \right\}$  Serum concentration of  $\left\{ \begin{matrix} \text{Sodium} \\ \text{Potassium} \\ \text{Urea} \end{matrix} \right\}$  milli-osmols per liter (mosM/l.)
2.  $\left. \begin{matrix} (F)_{Na} \\ (F)_K \\ (F)_u \end{matrix} \right\}$  Concentration of the respective substances in the glomerular filtrate.

Because corrections for serum water were not made, a small systematic error has been introduced into all calculations. The filtrate concentration of non-electrolytes was considered to be identical to serum. An average Donnan correction of 5 per cent was used for electrolytes.

3.  $\left. \begin{matrix} (U)_{Na} \\ (U)_K \\ (U)_u \end{matrix} \right\}$  Urine concentrations of sodium, potassium and urea etc., mosM/l.

<sup>3</sup> It is probable that the 50% urea infusion did not cause hemolysis because it was administered along with a large volume of the sustaining saline infusion.

4.  $V$  — minute urine volume (cc/min.), clearance of water ( $C_w$ ).
5.  $\frac{(U)_x V}{(F)_x}$  — Renal clearance of substance X, ml. of plasma or serum per minute.
6.  $C_x/C_{cr}$  — ratio of the simultaneously determined clearance of substance X to the creatinine clearance, the latter being used as a measurement of glomerular filtration rate ( $C_f$ ).
7.  $C_{cr} - V = W_R$ , water reabsorbed, ml. per minute.
8.  $(F)_x C_{cr} - (U)_x V = X_R$ , amount of substance X reabsorbed per minute expressed in mosm.
9.  $X_R/W_R = (R)_x$  concentration of substance X in reabsorbate, mosm/l.
10.  $[(F)_{Na} \times 2] + (F)_u = (F)_{osM}$ , concentration in the filtrate of substances exerting an osmotic pressure, mosm/l.
11.  $[(U)_{Na} \times 2] + (U)_u = (U)_{osM}$ , total concentration of substances in the urine exerting an osmotic pressure, mosm/l.

In reference to equations 10 and 11, the total osmotic pressure has been calculated as twice the sum of the Na concentration plus the urea concentration. Certain small errors have been neglected, namely 1) filtrate concentration of glucose and other non-electrolytes; 2) serum concentration of water; 3) concentrations of cations other than Na;<sup>4</sup> 4) concentrations of non-monovalent anions. Factors 1 and 2 were essentially constant. Factors 3 and 4 contribute a very small moiety to the total osmotic pressure of the urine during osmotic diuresis. The osmotic pressure as calculated, therefore, gives a first approximation of sufficient accuracy to warrant the calculations and conclusions made.

12. The amount of sodium reabsorbed per 100 ml. of glomerular filtrate was calculated:  $\frac{Na_R}{C_{cr}} \times 100 = Na_R/100 C_{cr}$

## RESULTS AND DISCUSSION

The observations reported are based on 15 experiments comprising 123 clearance periods.

**Glomerular Filtration Rate.** Control  $C_F$  averaged 4.9 cc/min/kg. body weight, with a range from 3.0 to 7.0. This does not differ significantly from the mean normal value reported by Houck (9) of 4.29 cc/min/kg. body weight with a range of 2.15 to 8.32, as determined in trained unanesthetized dogs. Although a slight fall in  $C_F$  resulting from the anesthesia and surgical trauma may have been masked by the priming dose of hypertonic saline, it is apparent that  $C_F$  was essentially normal at the start of each experiment. Values of  $(S)_u$  up to 100 mosm/l. were associated with no change in  $C_F$  (average change — 2 per cent), whereas at higher levels a decrease in  $C_F$  was observed. Although the depression of  $C_F$  was not studied specifically, it seems reasonable to attribute it to a fall in cardiac output and renal plasma flow due to toxic concentrations of urea. Dehydration was not a factor as urine volume was balanced by the infusion of equal amounts of isotonic saline.

**Osmotic Pressure of the Urine.** Priming with hypertonic saline resulted, with but a single exception, in a hypertonic urine referable to  $(U)_{Na}$  as well as  $(U)_{osM}$ . Urea diuresis produced a uniform pattern of response. As would be expected the  $(F)_{osM}$  progressively rose during the course of the urea infusion. However, during the initial periods of urea administration the  $(U)_{osM}$  fell to become essentially isosmotic with the filtrate. Thereafter,  $(U)_{osM}$  and  $(R)_{osM}$  rose with the  $(F)_{osM}$  and all three remained

<sup>4</sup> K was originally included in this calculation, but subsequently was not considered because of the undetermined nature of the mechanism of K secretion. The exclusion of K from the calculation does not alter the results significantly.

essentially isosmotic (fig. 1). In the final periods of 6 experiments the urine became hypo-osmotic to the filtrate. Definite conclusions cannot be drawn in the absence of direct measurements of osmotic pressure, but the data obtained suggest that hypo-osmotic urines can result from osmotic diuresis associated with an abnormally high osmotic pressure in the serum and presumably maximal posterior pituitary activity. To explain this, it may be postulated that the reabsorption of osmotically active agents occurs at a faster rate than the reabsorption of water, and that at high rates of urine flow insufficient time has elapsed for the slower process to reach osmotic equilibrium.

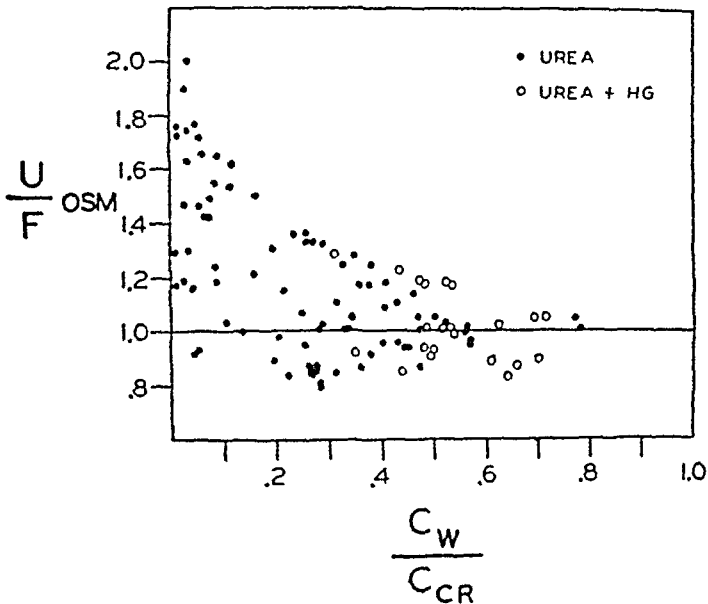


Fig. 1. EFFECT OF UREA DIURESIS on ratio of osmotic pressure of urine to plasma.

The production of hyperosmotic urines requires the tubular reabsorption of water in excess of solute, and calculations were made to define this quantity expressed as water reabsorbed without solute. The average values were 1.8 cc. per minute for the control periods, and 2.9 cc. per minute for the periods of maximal diuresis, all hypo-osmotic urines being excluded from the averaged values. As the total osmotic pressure rises, the analytical error is magnified in the calculations as presented, and the difference between the control and diuresis values cannot be considered significant. It is evident, however, that at normal or low rates of urine flow the production of a maximally concentrated urine would require the reabsorption of only small amounts of water free of solute. This limited capacity of the distal tubule would explain the formation of essentially isosmotic urines in the presence of large osmotic loads. Similar conclusions have been drawn from observations made in man during hydropenia (10) and osmotic diuresis (11). Such a calculation of distal function assumes that proximal reabsorption is isosmotic. The fact that essentially isosmotic urines were produced under the conditions of these experiments is taken as experimental evidence of the validity of this concept and is in agreement with the results obtained by the micro-puncture technique (12).

**Electrolyte Excretion.** The renal excretion of  $\text{Na}^6$ ,  $\text{K}^6$  and  $\text{Cl}$  were studied in all experiments. The results of two representative experiments are given in table 1 and figure 2. A uniform pattern of response was observed in every experiment. In the control periods  $(\text{U})_{\text{Na}}$  was hypertonic to  $(\text{F})_{\text{Na}}$ . As diuresis progressed  $(\text{U})_{\text{Na}}$  fell below

TABLE 1A

PERIOD	C <sub>F</sub>	V	W <sub>R</sub>	PER CENT REABSORBED				Na REAB- SORBED mEq/ 100 ml. C <sub>F</sub>	U/F OSMO- LAR RATIO	H <sub>2</sub> O REAB- SORBED WITHOUT SOLUTE
				H <sub>2</sub> O	Na	Cl	Urea			
	cc/min.									cc/min.
1	72.6	3.9	68.7	94.6	92.7	92.0	15.9	13.7	1.49	1.9
2	80.0	4.0	76.0	95.0	92.2	91.0	18.7	13.4	1.68	2.7
40% urea infusion started at 2 ml/min.										
3	71.7	6.0	65.7	91.5	91.2	90.3	5.7	13.3	1.57	3.4
4	66.3	14.0	52.3	78.9	85.3	81.3	5.8	12.7	1.12	1.7
5	71.3	22.9	48.4	68.5	74.7	71.0	10.0	11.7	1.09	2.1
6	70.6	28.7	41.9	59.6	66.8	63.7	7.9	10.6	1.09	2.6
7	66.2	31.1	35.1	53.1	63.6	59.8	5.3	10.2	1.06	1.9
8	61.0	28.8	32.2	52.8	65.6	62.9	12.5	10.6	0.99	-0.2

Dog 1. 18.0 kg. Consecutive clearance periods of 20 minutes each. For abbreviations see text.

TABLE 1B

PERIOD	FILTRATE				URINE				REABSORBATE			
	Na	Cl	Urea	Total <sup>1</sup>	Na	Cl	Urea	Total	Na	Cl	Urea	Total
	mosM/l.				mosM/l.				mosM/l.			
1	148	133	2.6	299	202	195	40	444	145	129	0.13	290
2	145	131	2.6	294	226	210	42	494	141	126	0.51	283
3	145	131	16.8	307	147	149	187	481	145	129	1.04	291
4	149	137	38.1	336	104	122	170	378	161	141	3.77	326
5	156	145	53.7	366	123	130	151	397	172	151	7.85	352
6	158	149	72.0	388	129	133	164	422	178	160	9.53	366
7	160	151	89.0	409	123	128	179	425	192	170	8.80	393
8	162	155	100.0	424	118	122	186	422	201	185	23.6	426

<sup>1</sup> See text for calculation of total osmotic pressure.

$(\text{F})_{\text{Na}}$  and more of the filtered water than of the filtered sodium was excreted. When the results are calculated in terms of the reabsorbate it is seen that  $(\text{R})_{\text{Na}}$  exceeded  $(\text{F})_{\text{Na}}$  and that the difference in these concentrations,  $(\text{R}-\text{F})_{\text{Na}}$ , was approximately proportional to  $(\text{S})_{\text{u}}$  (fig. 3). Individual experiments showed a more linear relationship than indicated in figure 3 and the scatter of the massed plots may be attributed to

<sup>6</sup> Throughout this paper the excretion of Na and accompanying anion will be presented in terms of Na alone without the implication that the reabsorptive mechanism for Na is primary and for anion secondary.

<sup>6</sup> The studies of potassium revealed a tubular secretory mechanism. A preliminary paper has been published (13). Further studies are in progress and will be reported subsequently.

variations in the degree of urine hypertonicity and the extent to which urea was reabsorbed.

The ability of the kidney to produce reabsorbate that is hypertonic with respect to Na has been attributed to the absence of the antidiuretic hormone (14), but was subsequently noted with osmotic diuresis (15). The present experiments extend these

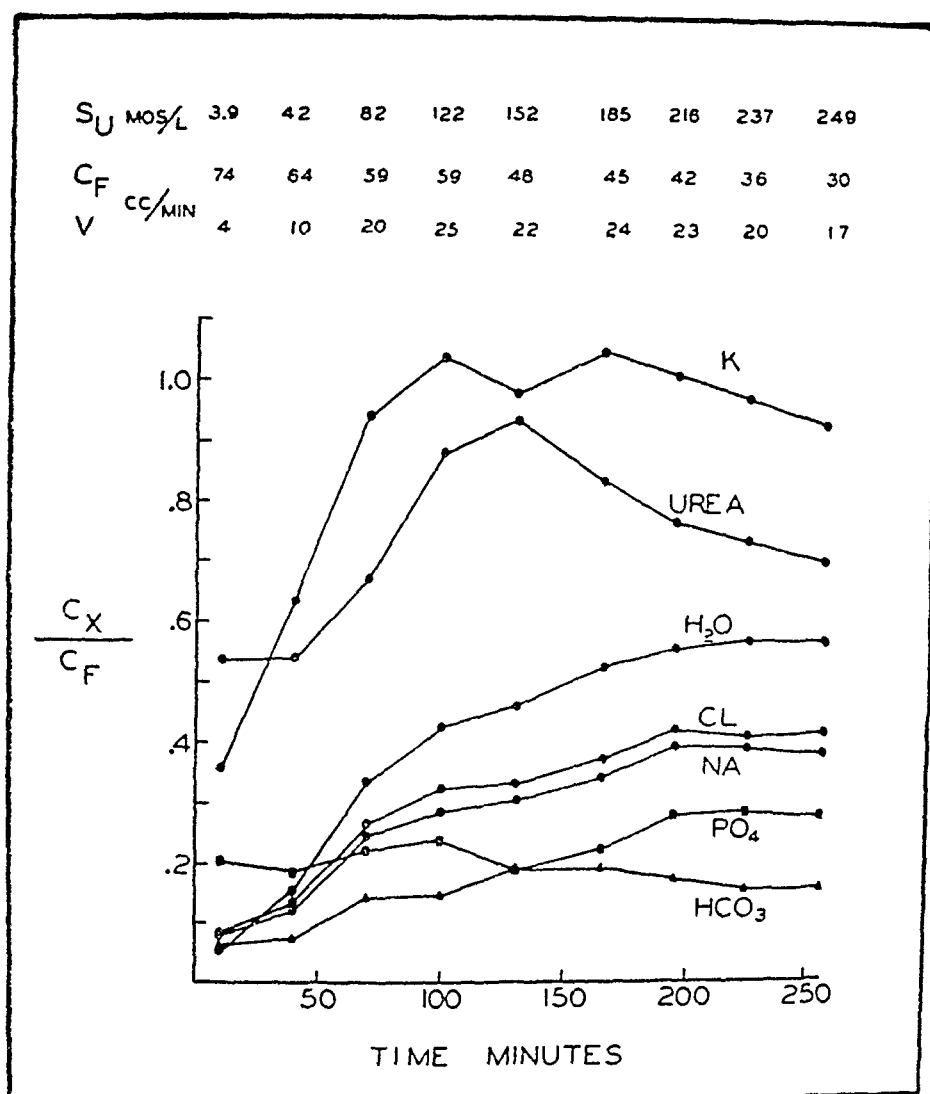


Fig. 2. ELECTROLYTE CLEARANCES during urea diuresis.

observations to higher rates of excretion, and, as subsequently discussed, localize the process to the proximal tubule. The reabsorption of Na can therefore occur against a concentration gradient, indicating that Na is reabsorbed by an active metabolic process and not by diffusion in response to concentration gradients established by movements of water. The fact that the filtrate, reabsorbate and urine became essentially isosmotic in the presence of marked differences in the respective concentrations of osmotically active components indicates that water is reabsorbed by back diffusion under the conditions of these experiments.

Previous calculations (16) have suggested that the major portion of the glomeru-

lar filtrate is reabsorbed in the proximal tubule and a variable percentage of the remainder is reabsorbed more distally. It is obvious that the magnitude of osmotic diuresis is such that any simple percentile analysis of proximal and distal functions is

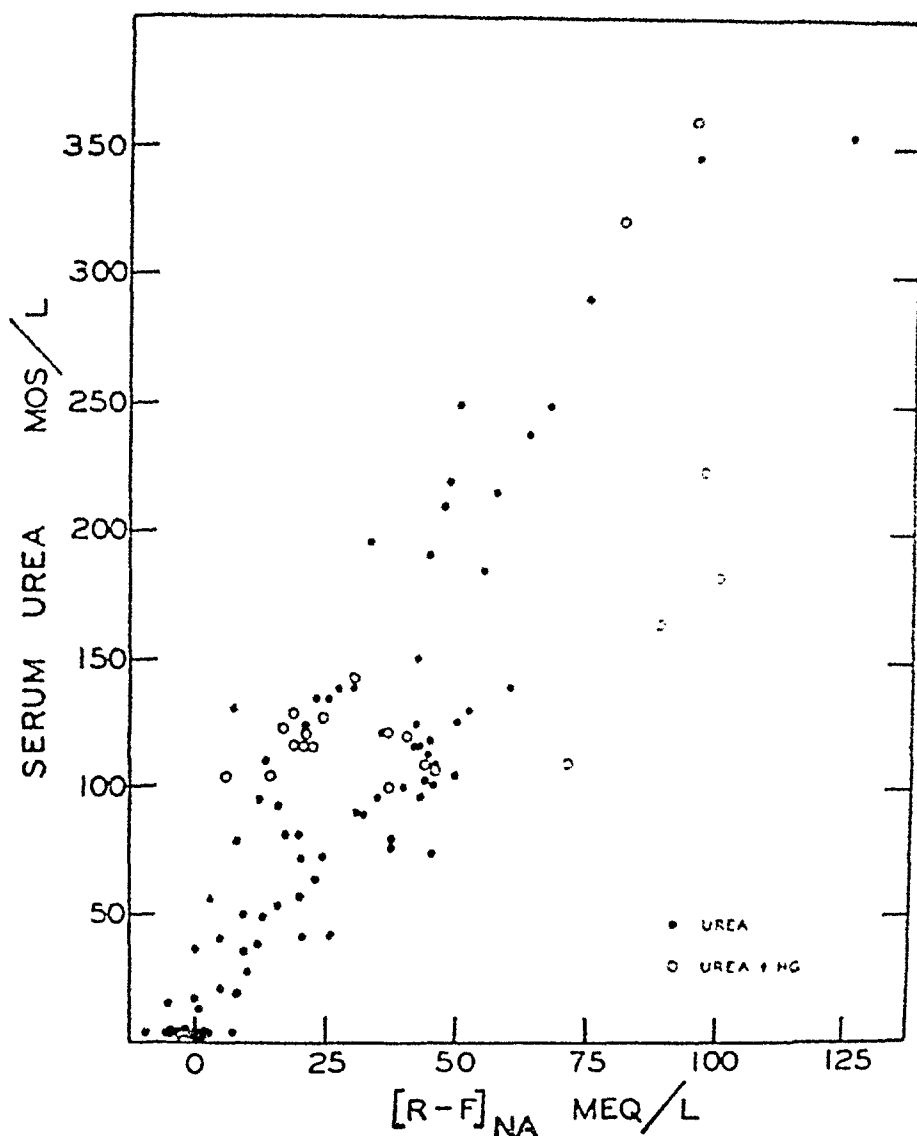


Fig. 3. RELATIONSHIP OF CONCENTRATION of sodium in reabsorbate and filtrate to serum urea.  $R-F_{Na}$  is the sodium concentration in the reabsorbate minus the sodium concentration in the filtrate. The relationship obtained during urea diuresis is not significantly altered by the administration of a mercurial diuretic.

impossible. The important question remains as to whether or not diuresis per se produces so large an intersegmental imbalance that distal function becomes ineffective in absolute terms. A relative diminution of distal function is evident from the above data on osmotic pressure which showed that the volume of water removed free of solute remains small and essentially constant despite wide variations in urine flow. The relative importance of this reabsorption gradually decreases as urine volume increases and voided urines become isosmotic. If a similar reasoning is applied to

the reabsorption of Na, it may be said that, even though the total reabsorptive capacity of the distal tubule is unknown, its relative importance in determining the characteristics of the voided urine certainly decreases with forced diuresis. It is therefore reasonable to attribute the phenomena observed to activities of the more proximally located tubular cells. This interpretation is in agreement with the independent observations of Wesson and Anslow on mannitol diuresis (2). However, it must be emphasized that this concept represents only a reasonable interpretation of the available data in as much as there is no conclusive proof that distal activity has not changed in the presence of increased loads.

The question remains as to why the absolute amount of excreted sodium increases under the influence of a non-electrolyte osmotic diuretic. If sodium is actively reabsorbed proximally, why doesn't this process remove essentially all the Na from the proximal urine, delivering an isosmotic fluid to the distal tubule with urea as the major osmotic component? The increased Na excretion does not result from an increase in  $(S)_{Na}$ . In the above experiments  $(S)_{Na}$  was normal or elevated and in the experiments of Wesson and Anslow a similar increase in Na excretion was observed while  $(S)_{Na}$  was falling sharply. A specific nephrotoxic action of urea seems unlikely because of the comparable effects of glucose (14, 17, 18) and mannitol, and because the effect of urea was a graded response developing with relatively slight elevations of  $(S)_u$ .

An insight into the possible mechanisms whereby the reabsorption of Na is diminished is obtained by a comparison of saline and urea diuresis. In table 2 are depicted the results of a pair of experiments in which final  $C_F$  and  $(F)_{Na}$  are comparable. The amount of Na reabsorbed, expressed as mEq/100 ml. of filtrate, remains relatively constant with saline but decreases sharply with urea diuresis. In saline diuresis Na (and accompanying anions) is the major component contributing to the osmotic pressure of the filtrate. Therefore as Na is actively reabsorbed and water back diffuses the Na concentration of the tubular urine remains essentially unchanged. In urea diuresis the Na concentration of the filtrate, although the same in absolute terms, contributes a smaller moiety to the total osmotic pressure. Therefore when Na is reabsorbed less water back diffuses to maintain osmotic equilibrium. Consequently the concentration of Na in the reabsorbate is higher than that of the filtrate and the Na concentration in the tubular urine falls progressively along the length of the proximal tubule. This suggests the possibility that the rate of reabsorption may be partly determined by the concentration of Na in the tubular urine and by the length of time that a given concentration prevails.

Wesson and Anslow (2) reached a somewhat similar conclusion from their studies, but believed that the proximal reabsorption of Na was arrested by a limiting concentration gradient. This gradient was obtained when the difference between plasma and urine Na concentration,  $(F - U)_{Na}$ , reached a value of 60 to 90 mEq/l. We do not believe that the results of forced diuresis experiments warrant this conclusion for the following reasons. 1) There is no apparent upper limit to the concentration differences developed between filtrate and reabsorbate (Fig. 3). 2) The value of  $(F - U)_{Na}$  obtained in our experiments, although relatively constant for short time intervals, varied widely in single experiments and from animal to animal. 3) During

periods of maximal diuresis it was evident that this value varied inversely with fluctuation in  $C_F$ , indicating that with lowered Na loads more complete reabsorption of Na occurred even in the presence of isosmotic urines. 4) During osmotic diuresis tubular reabsorption of Na appears to be reduced by the exposure of a large segment of the tubule to urine of lowered Na concentration.

The experiments on forced diuresis obviously do not define the relative reabsorptive capacities of the proximal and distal tubule under normal conditions. Nevertheless, the results certainly do not invalidate the possibility that almost complete reabsorption might occur proximally under conditions of reduced load, under

TABLE 2. COMPARISON OF SODIUM REABSORPTION DURING UREA AND SALINE DIURESIS

PERIOD	C <sub>F</sub>	V	(S) <sub>u</sub>	(F) <sub>Na</sub>	(U) <sub>Na</sub>	U <sub>Na</sub> V	Na REAB- SORBED/ 100 ML. C <sub>F</sub>
	ml/min.		millimoles/l.			mEq/min.	mEq.
	Urea Diuresis						
1	94.8	8.2	3.4	149	215	1.76	13.0
2	100.0	11.4	3.5	152	221	2.52	12.7
	Infusion 50% urea, 2 ml/min.						
3	107.3	16.8	15.7	152	182	3.05	12.4
4	106.2	24.5	37.6	149	148	3.61	11.4
5	100.0	29.4	56.3	148	140	4.11	10.6
6	99.6	32.8	77.8	148	133	3.45	10.5
7	97.4	34.0	94.8	149	127	4.31	10.5
8	92.4	34.9	110.8	150	128	4.45	10.2
	Hypertonic Saline						
1	58.9	.40	4 <sup>1</sup>	139	14	.006	13.9
2	69.1	.59		139	23	.014	13.9
	Infusion NaCl 900 mEq/l., 2 ml/min.						
3	69.6	.90		143	58	.052	14.2
4	69.9	2.05		143	119	.243	13.8
5	82.5	3.90		147	154	.600	14.0
6	83.9	5.10		146	168	.857	13.5
7	85.4	5.85		146	177	1.035	13.4
8	90.4	5.33		148	185	.983	13.8

<sup>1</sup> Assumed value. For abbreviations see text.

which circumstances the lower portions of the proximal tubule might be exposed to small volumes of residual tubular urine of low Na concentration and the isotonicity of the urine might be maintained by other un-reabsorbed components of the filtrate, mainly urea.

*Miscellaneous Observations.* The following incidental observations are briefly reported, all values being given for periods of maximum diuresis. In the 4 experiments in which measurements were made there was no evidence of glucose appearing in the urine when the urine volume was as much as 53 per cent of the filtrate. Urine pH averaged 6.9 in one experiment in which urines were collected anaerobically, and approximated 7 in 4 other experiments. Bicarbonate clearance averaged 14 to 19 per cent of

$C_F$  in one experiment, serum bicarbonate being 17.4 to 21 mEq/l. Low bicarbonate clearances have been reported in mannitol diuresis which also were associated with low serum bicarbonate concentrations (2). Phosphate clearances varied from 18 to 29 per cent and from 22 to 38 per cent of  $C_F$  in two experiments, in both instances being associated with an elevation of serum phosphorus to approximately twice the control value. In a third experiment the urine became phosphate free as diuresis increased, serum phosphate remaining constant. Chloride determinations were done in all experiments, and  $C_{Cl}$  paralleled  $C_{Na}$  in all periods.

Inasmuch as all animals were studied under the influence of a NaCl load, a mild high-chloride acidosis must have existed in each experiment. Under these conditions, the high excretion of chloride and the formation of a urine more acid than plasma are not unexpected. The low clearance of bicarbonate was associated with an isosmotic urine, suggesting that bicarbonate reabsorption in relative excess of water and chloride reabsorption occurred in the proximal tubule. This interpretation is compatible with observations obtained from the micropuncture studies of mammalian tubules (12) in which a chloride U/P ratio of 1.4 was found in the first half of the proximal tubule.

*Effect of Mercurial Diuretics.* In 6 experiments mercurial diuretics<sup>7</sup> were administered during urea diuresis. Due to the concomitant variations in  $C_F$ ,  $(S)_{Na}$  and  $(S)_u$  a quantitative analysis of the mercurial action can not be obtained. However, in every instance the mercurials markedly increased  $(U)_{Na}$  and  $(U)_{Na}V$ . In the experiment depicted in figure 4  $(S)_{Na}$  and  $(S)_u$  were relatively constant and  $C_F$  fell slightly. Despite the decrease in sodium load, the effect of the mercurial on the reabsorption of sodium is obvious. Considering these results in terms of the previously stated premise that during severe osmotic diuresis the relative role of the distal tubule is small, the predominant action of the mercurials appears to be on the proximal tubule. The mercurial diuretics do not significantly effect  $(R - F)_{Na}$  as shown in figure 3. This indicates that in the presence of a mercurial diuretic the process of the proximal tubular reabsorption of sodium remains one of active transfer, but reabsorptive capacity is depressed.<sup>8</sup>

*Urea Clearance.* Previous studies have shown that urea is excreted by filtration and back diffusion (19). The present observations, made at high serum urea concentrations, are in essential agreement with this concept. An analysis of those periods in which  $C_F$  is maintained within 85 per cent of the control value shows that the clearances of urea and water increase concomitantly. The massed data fall on a curve almost identical to that published by Shannon (19). In prolonged experiments with declining  $C_F$ , however, this relationship does not persist,  $C_u/C_F$  falling progressively despite a further decrease in the U/P creatinine ratio. This may be seen

<sup>7</sup> 1 or 2 ml. of 'Mercupurin' or 'Salyrgan' were given intravenously. We are indebted to Dr. M. L. Tainter of the Winthrop Chemical Co. for the 'Salyrgan' used in these studies.

<sup>8</sup> Since this manuscript was submitted, DUGGAN, J. J. and R. F. PRITS (*Federation Proc.* 8: 37, 1949) have presented evidence that mercurials, given during a saline diuresis, inhibit the reabsorption of a maximum of 1500  $\mu$ Eq of Na per minute, or about 16% of the amount filtered, indicating a distal site of action. In 2 experiments with urea diuresis, in which the filtered loads of both Na and urea remained constant, the increase in Na excretion was 2550 and 2130  $\mu$ Eq/minute, representing 21 and 28% of the filtered load. It is evident that the apparent maximal effect of mercurial diuretics may depend upon the experimental conditions.

in figure 1. Presumably, when decreased amounts of filtrate are delivered to each nephron there is an increase in the duration of time tubular urine remains in contact with the tubular cells. This would enable more urea to diffuse back even though the progressive fall in the U/P creatinine ratio results in a decreasing concentration gradient referable to urea. The increased reabsorption of urea at high  $(S)_u$  and lower  $C_F$  values contributes significantly to the osmotic pressure of the reabsorbate and lowers the value  $(R - F)_{Na}$ . This partially explains why the plots in figure 3 deviate from a linear relationship.

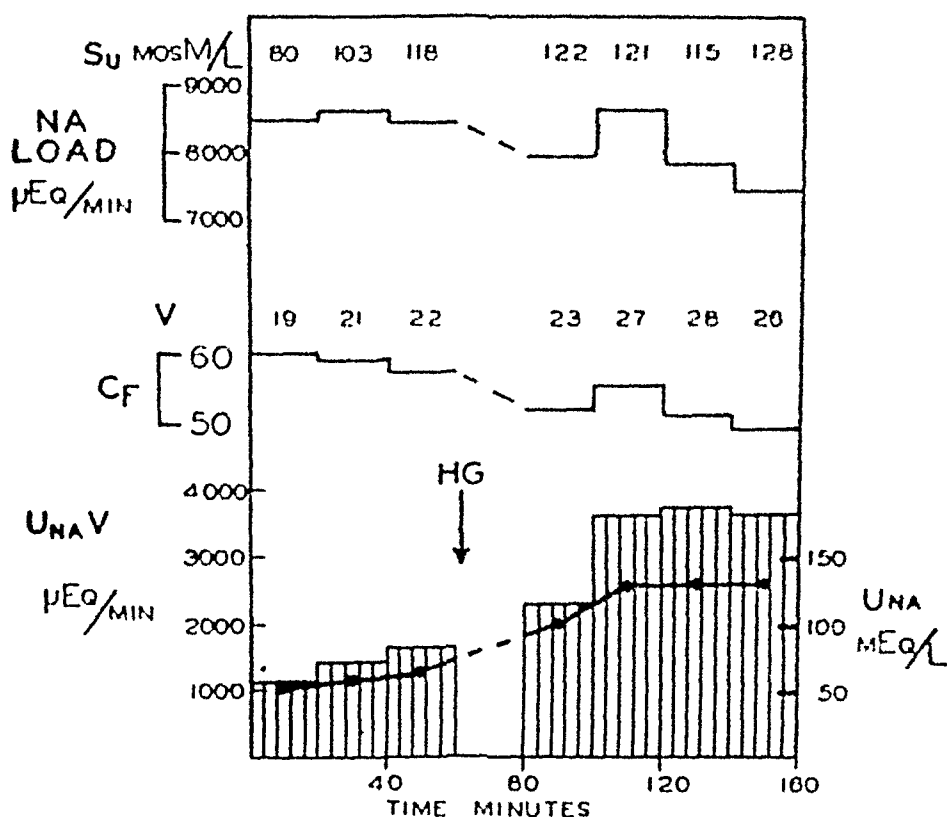


Fig. 4. EFFECT OF ADMINISTRATION of a mercurial diuretic during a urea diuresis showing an increased sodium excretion with constant serum urea levels and slightly falling filtered sodium loads.

*Renal Work.* The conventional calculations of renal work have followed a strict application of thermodynamic principles (11, 20, 21). These consider the initial plasma concentration of a given substance, the final urine concentration, and the amount excreted per unit time, and state that energy is expended by the kidney if the urine concentration of a given substance is higher than that of the plasma, and conversely energy is made available to the kidney if the urine concentration is less than that of the plasma. The paradox of measuring the work of certain biological processes in thermodynamic units becomes apparent when renal reabsorptive mechanisms are analyzed. By far the greatest part of Na reabsorption is isosmotic and the U/P ratio of Na remains essentially unchanged. Therefore to satisfy a thermodynamic equilibrium the energy expended in the active transfer of Na would have to be regained by the passive diffusion of water. This would appear to be unreasonable. An additional

inconsistency is revealed by an analysis of the above experiments. Na was excreted in a lower concentration than it exists in the plasma and according to thermodynamic concepts there was a gain in energy and the kidney was doing no work referable to Na excretion. However, if the tubular process is examined it is seen that Na was reabsorbed from the low concentration of the tubular urine to either the high concentration of the plasma or the even higher concentration of the reabsorbate. In either case the tubular cells were transferring Na from a low concentration to a higher one and thus performing work referable to Na excretion. Therefore the kidney either gained or consumed energy in excreting Na depending upon the point of view from which the same process is analyzed. It is obvious therefore that the analysis of the work of the kidney in terms of initial and final solute concentrations yields results which are misleading and are quite unrelated to the observed biological phenomena.

#### SUMMARY AND CONCLUSIONS

Extreme degrees of osmotic diuresis were produced in anesthetized dogs by the intravenous infusion of a solution of 50 per cent urea sufficiently rapidly to produce plasma concentrations of urea in excess of 100 mosm/l. within a period of a few hours. Water balance was maintained by infusing solutions of NaCl at a rate commensurate with urine flow. By this technic glomerular filtration rate was maintained at or near control levels until depressed by inordinately high concentrations of urea. The animals showed the following responses:

Urine flow progressively increased and in many experiments reached 50 per cent of the glomerular filtration rate. At high rates of flow the urine became approximately isosmotic with the blood which indicated the functional incapacity of the distal tubule to alter significantly the composition of the large volume of filtrate it received. Thus differences between the composition of the glomerular filtrate and the formed urine were interpreted as largely the result of activity of the proximal tubule.

The tubular reabsorbate was isosmotic and, since only small amounts of urea were reabsorbed, sodium salts provided the major osmotic constituent of the reabsorbate. This resulted in a sodium concentration of the reabsorbate greatly in excess of that of the filtrate or plasma. A concentration gradient relative to sodium between proximal tubular filtrate and reabsorbate indicates an active transfer of sodium by proximal tubular cells. The isotonicity of the formed urine is readily explained by the back diffusion of water due to the osmotic gradient created by the transfer of sodium.

The presence of urea in the proximal tubule greatly diminishes the reabsorption of sodium even though the sodium load remains constant. Arguments are presented which favor the view that total sodium reabsorption is decreased because of the continuous fall in proximal tubular sodium concentration as each increment of the glomerular filtrate is reabsorbed, a condition which only applies in the presence of an osmotic diuretic.

The administration of a mercurial diuretic at the height of urea diuresis further decreases the tubular reabsorption of sodium but does not alter the concentration gradient relative to sodium between tubular urine and reabsorbate. This indicates that mercurial diuretics decrease the functional capacity of the proximal tubular

cells actively to transfer sodium but do not alter the fundamental mechanisms of sodium reabsorption.

The data obtained on the renal clearance of urea at high plasma levels are in keeping with the view that the excretion of urea is a process of filtration and passive back diffusion. The problem of renal work is discussed in relation to the experimental results.

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# CAPILLARY PERMEABILITY: RATE OF TRANSCAPILLARY EXCHANGE OF CHLORIDE IN THE GUINEA PIG AS DETERMINED WITH RADIOCHLORIDE

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WE HAVE previously reported on the relative rate of transcapillary exchange in the guinea pig of water (1), sodium (2), and iron (3). The present paper deals with the rate of transcapillary exchange of chloride using radiochloride as the tracer substance. Beyond adding to our knowledge of the relative permeability of the capillary wall to the substances of the plasma, we believe that the data can be used to test an interesting and stimulating hypothesis proposed by Chambers and Zweifach (4, 5). This hypothesis states that an electrolyte passes from the plasma to the extravascular fluid in major part through the intercellular cement of the capillary rather than through the total capillary wall or through the endothelial cell, whereas water and dissolved gases pass through the whole wall.

## METHODS

Ten adult guinea pigs were used for the experiments. Sodium radiochloride was injected into the vein of a foreleg and samples of blood were subsequently obtained under light nembutal and ether anesthesia from a cannula placed in the proximal end of a carotid artery. To prevent shift of chloride from red cells to plasma, the following precautions were taken to avoid loss of  $\text{CO}_2$  during collection of the samples and separation of the plasma. The cannula in the carotid artery was filled with oil prior to sampling and all the blood samples drawn into oiled syringes. The blood was then delivered under oil into centrifuge tubes containing heparin and promptly centrifuged. Plasma rather than whole blood was used for radioactivity measurements because of greater ease in handling and measuring.

Radiochloride ( $\text{Cl}^{38}$ ) was prepared from KCl by deuteron bombardment in the cyclotron of the Carnegie Institution of Washington. The KCl was dissolved from the target with a small amount of water and the solution filtered into a large centrifuge tube. A solution of  $\text{AgNO}_3$  and a few drops of concentrated  $\text{HNO}_3$  were then added. After stirring, the precipitate was allowed to settle and a drop of  $\text{AgNO}_3$  added to test for complete precipitation. The supernatant fluid was decanted and the precipitate washed 4 times with small portions of water. The precipitate was then covered to a depth of 2 or 3 centimeters with 0.01 N HCl and an excess of granular zinc added. Using the bottom of a test tube as a pestle, the zinc and AgCl were ground together.

Although reduction of the silver was exothermic, room temperature was satisfactory for the reaction. Zinc was precipitated from the soluble  $\text{ZnCl}_2$  as the carbonate by adding powdered  $\text{Na}_2\text{CO}_3$  in the presence of a few drops of cresol red. The carbonate was added until the indicator turned red.  $\text{ZnCO}_3$  was then centrifuged down and the solution of sodium radiochloride decanted off. After determining the chloride content of this solution, it was diluted to isotonicity.

Half a milliliter of plasma was measured onto filter paper disks placed at the bottom of shallow lucite cups of uniform dimensions. A small amount of detergent was then added; this and the filter paper insured uniform distribution of the samples in the cup. To eliminate errors due to unequal evaporation among the samples, each was prepared just prior to its measurement. Because of the rapid rate of decay, it was necessary to use for decay correction the mean time of the interval during which each sample was measured. This became more important in the later samples of a series where increased measuring time was necessary for equal accuracy of all determinations. Aliquots of a standard solution were measured intermittently with the plasma samples. These permitted decay corrections to be made with ease and also provided an immediate conversion factor for the activity of each sample into concentration of labeled chloride per ml. plasma. In addition, since the decay curve of the standard proved to be that of  $\text{Cl}^{35}$ , we were assured of the reliability of the measuring technique and of the absence of radioactive contaminants. Only 4000 counts were taken for each sample which results in a standard deviation of about 2 per cent. However, after an initial determination with Geiger counter and scaling circuit, all samples were checked with electrometer and ionization chamber.

## RESULTS

Since we have been interested in the average rate of exchange of plasma chloride with extravascular chloride rather than its value in a single animal, the measurements on the 10 animals have been placed on a common basis. This has been accomplished by adjusting the amount of chloride given each animal to a standard amount per kilogram body weight. The average of the several concentrations of chloride used in the experiments was 39.49 mg/kilogram body weight; this quantity has consequently been used as the standard quantity. The intensity of radioactivity, as well as the quantities of chloride injected, is considered to have been so low as to have caused no abnormal effects.

The change of concentration of radiochloride in the plasma with respect to time after intravenous injection is shown in figure 1. The curve has been fitted to the points by inspection. As shown in the insert of figure 1, when the logarithm of the concentration in the plasma in excess of the equilibrium concentration is plotted against time, the points fall about a straight line. This means that the concentration of the plasma radiochloride approaches equilibrium at a constant rate and that the disappearance curve is described by the equation previously derived for water and sodium (1, 2):

$$C_t - C_{eq} = (C_0 - C_{eq})e^{-R/q \cdot t} \quad (1)$$

where  $C_t$ ,  $C_0$  and  $C_{eq}$  are the concentrations of radiochloride in the plasma, at time,  $t$ , at  $t = 0$  and at equilibrium respectively;  $R$  is the proportion of the normally occur-

ring chloride of the plasma which exchanges per unit time with extravascular chloride and  $q$  is the proportion of the total chloride which is not contained in the plasma but is in red cells and extravascular fluid.

Unlike sodium, a considerable amount of radiochloride can be demonstrated in the red cells of the guinea pig. We have measured the partition coefficient of radiochloride between plasma and red cells of the guinea pig and found it to equal 2.17, a value essentially the same as that for man and the rabbit (6). As might have been predicted from other observations, chloride enters the red cell from the plasma with

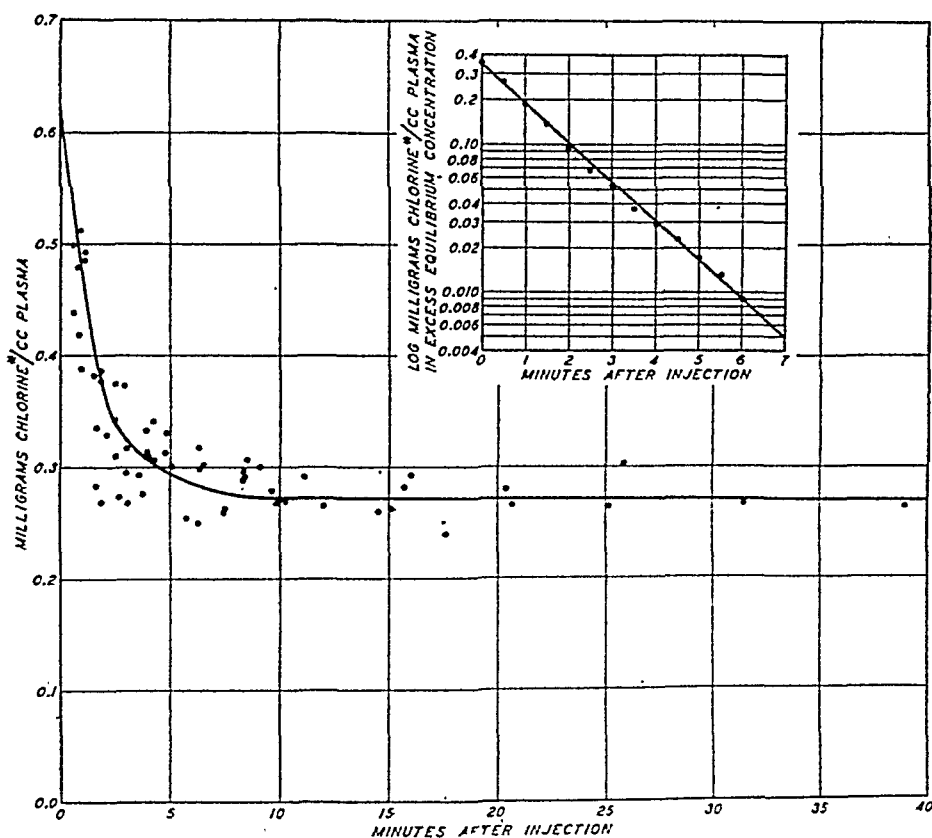


Fig. 1

great rapidity. The partition coefficient remains constant whether the plasma is separated by centrifugation immediately after radiochloride is added to whole blood *in vitro* or whether it is separated after an hour or so has elapsed.

The radiochloride present in the red cells must be considered in evaluating the constants of equation 1. Since radiochloride rapidly exchanges between plasma and red cells, red cells and plasma are considered to have been at equilibrium in all samples of blood from which plasma was separated for measurement of radioactivity. On the assumption of a hematocrit of 50, the partition coefficient of 2.17 means that 32 per cent of the radiochloride of whole blood is present in the red cells. The concentration of radiochloride in the plasma at  $t = 0$ , as given by the extrapolation of the logarithmic plot of the insert of figure 1, is 0.633 mg/ml. of plasma (zero value from graph plus equilibrium value), where  $C_{eq} = 0.273$  mg/ml. of plasma. This is in excellent agreement with the value of  $C_0$ , 0.645 mg/ml. of plasma, calculated from the initial

concentration of radiochloride (39.49 mg./kilogram body weight), the amount of plasma per kilogram body weight (43 ml.; 1) and the percentage of the radiochloride present in the red cells. As previously stated, the constant  $q$  is the proportion of the total chloride which is contained in both the extravascular fluid and the red cells. If  $C_0' =$  mg. of radiochloride injected per kilogram of body weight (39.49 mg.) divided by the volume of plasma per kilogram body weight (43 ml.) = 0.918 mg. radiochloride per ml. plasma; then  $C_{eq} = C_0'(Cl_p/\text{total Cl})$ . By the derivation previously given (2),  $q$  can be obtained from the equation  $C_{eq} = C_0'(1 - q)$ . The value of  $q$  from this equation is 0.702.

The slope of the logarithmic plot of the insert of figure 1 is equal to  $-0.63$  which is the value of  $-R/q$ . Substituting the value 0.70 for  $q$ ,  $R$  is found to equal  $-0.44$  which means that 44 per cent of the plasma chloride is exchanging each minute with extravascular chloride. In terms of the total chloride which escapes across the capillary wall, however, we must consider that lost from the red cells as well. This chloride at time of measurement is in equilibrium with that of the plasma and consequently

TABLE 1. CONSTANTS DESCRIBING RATES OF EXCHANGE OF CHLORIDE, SODIUM AND WATER BETWEEN VASCULAR AND EXTRAVASCULAR SYSTEMS

CONSTANTS	CHLORIDE	SODIUM	WATER
(1) Proportion of amount of plasma substance transferred to extravascular fluid per minute	.64	.60	1.46
(2) Loss in plasma concentration of tracer substance per minute relative to excess concentration (exponential rate)	.63	.73	.82

the chloride which crosses the capillary wall in an interval is equal to that lost from the plasma plus that lost from the red cells. The total rate of transcapillary exchange in terms of plasma chloride is therefore equal to  $R \times 1.46$  (the sum of chloride of plasma plus that of red cells divided by that of plasma) = 0.64, which states that an amount of chloride equal to 64 per cent of that in the plasma crosses the capillary wall each minute.

#### DISCUSSION

As shown in table 1, the rates of transcapillary exchange of sodium and chloride are equal when expressed in terms of the proportion of the amount in plasma transferred to the extravascular fluid per minute. This tells us within the limits of the method that the capillary wall of the guinea pig is equally permeable to sodium and chloride. Plasma water, however, is exchanged at the rate of 146 per cent of that of the plasma per minute and we can consequently say that the capillary wall is 2.3 times as permeable to water as to sodium and chloride.

As previously stated, Chambers and Zweifach postulate that water and dissolved gases pass through the whole of the capillary wall, whereas electrolytes escape in whole or in major part through the intercellular cement. We previously attempted to test this hypothesis quantitatively (10) and concluded that the whole of the capillary

wall is probably permeable to sodium and chloride as well as to water. This quantitative analysis was inaccurate because the difference in concentration across the capillary wall was used in Fick's formula for diffusion rather than the true concentration gradient. We arrive, however, at the same conclusion that the whole of the wall is most likely permeable to water, sodium, and chloride through qualitative consideration of our data. We shall analyze the postulate of Chambers and Zweifach on the basis of these assumptions: Following them, we assume that one per cent of the capillary wall is cement substance; secondly, we assume that the endothelial cell is 'freely' permeable to water as are other cells of the body; and finally, we suppose that the intercellular cement has approximately the same permeability to water as does the endothelial cell. It would be anticipated, if water escapes across the whole wall and electrolytes only through the cement, that water would cross the wall at a rate many times greater than that for sodium and chloride. In fact, one would expect that the rate of transcapillary exchange of water would be about 100 times greater than the rates for sodium and chloride, if the diffusion rates for these substances are approximately equal.

Hober (7) indicates the variation with molecular weight of the diffusion coefficients of a few common substances having molecular weights comparable to that of water. The maximum difference in these diffusion coefficients is not greater than a factor of 5. This means that following Fick's law

$$dN/dt = -Da(dc/dx)$$

the amount of substance freely diffusing across an inert membrane will be proportional to the diffusion coefficient  $D$ . If the area  $a$  and the concentration gradient  $dc/dx$  are considered equal in the vascular system of the guinea pig for sodium, chloride and water, the rate of exchange should not differ for these substances by more than the differences found in Hober's table, if diffusion is the main process by which these substances are transported across the capillary wall.

Our results with chloride, and earlier results with sodium and water, show that the rates for sodium and chloride ions are equal and that water is only about 2.3 times greater than these rates and not approximately 100 times greater as would be expected using the assumptions above.

Two types of objection might be made to this reasoning. It could be objected that diffusion might not be the sole process in the transport of chloride from the plasma to the extravascular fluid and that filtration would play a large role in the determination of the measured rates of exchange. The quantity of chloride, however, which moves across the capillary membrane from plasma to extravascular fluid is approximately equal to that moving from the extravascular fluid to the plasma. This latter process is considered to be purely diffusion as postulated by Starling (8). In addition it might be argued that a negligible amount of water passes through the endothelial cell and the major portion through the intercellular cement and that consequently the rates of turnover of plasma sodium, chloride and water are alike. This view, however, leads to the assumption of a permeability for the intercellular cement which is so high as to appear unreasonable to us. If only one per cent of the water passes through the endothelial cell, it can readily be shown that the intercellular

cement must be 10,000 times as permeable to it as the endothelial cell. If 10 per cent of the water passes through the endothelial cell, the cement must be 1000 times as permeable. We believe it more reasonable to assume that the permeability of the cement substance and endothelial cell to water are approximately equal and that consequently the whole wall must be permeable to water, sodium, and chloride to explain the similarity in their rates of turnover in the plasma.

The process of ultrafiltration through pores has been shown by Flexner (9) to be inadequate to account for significant separation of solvent from solute because the capillary hydrostatic pressure is insufficient to produce this separation to any appreciable degree. The observation that plasma water is exchanged more rapidly than sodium or chloride confirms the view derived from kinetic and thermodynamic considerations (10) that diffusion rather than filtration is the essential process in the exchange of substances across the capillary wall.

#### SUMMARY

The rate of turnover of plasma chloride has been determined by radio tracer techniques and compared to that previously reported on water and sodium. Sixty-four per cent of plasma chloride is exchanged each minute with extravascular chloride, while 60 per cent of the plasma sodium and 146 per cent of the plasma water is exchanged each minute. It is believed that the whole capillary wall is permeable to chloride, sodium, and water, and that chloride and sodium do not escape by way of the intercellular cement alone. Diffusion rather than filtration is concluded to be the predominant process in the exchange of substances across the capillary wall.

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# VOLUME ELASTIC PROPERTIES OF THE RIGHT AND LEFT ATRIUM<sup>1</sup>

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THE maintenance of a pressure gradient from the left to the right atrium in normal hearts and in hearts with atrial septal defects has been attributed by Opdyke and co-workers (1) and also by Cournand (2) and others (3), on the basis of indirect evidence, to a difference in the distensibility of the atria. While quantitative studies have been made of the elastic properties of veins (4-6), only quasi-volume elasticity curves for the right and left atrial systems have been published (1).

In view of the importance placed on the elastic properties of the two atrial systems in explaining the left to right shunt through an atrial septal defect (2, 3), it was thought advisable to re-investigate this problem using a quantitative approach.

## METHODS

The heart and lungs with a portion of the superior and inferior vena cavae were removed from dogs immediately after death. The lungs were carefully dissected away to preserve as much as possible of the main pulmonary veins. A glass cannula was tied into the superior vena cava and another into one of the larger pulmonary veins. The coronary sinus and all other vessels emptying into the atria were ligated. The walls of the ventricles were cut away and the tricuspid and mitral valves were sutured in the position of anatomical closure with fine silk on atraumatic needles. In some cases the valves were reinforced with wax to prevent leakage. The atria were filled with normal saline under low pressure and carefully checked for leaks. When none was found, the heart was submerged in a large container of saline as is shown in figure 1. The glass cannulae (A and B) were placed in such a position that they extended above the fluid level of the container. Saline was repeatedly introduced and withdrawn until all air was removed from the atrial cavities (R.A. and L.A.). The atria were then filled until the fluid level in the cannulae (X and Y) reached the same height as the level of the surrounding saline. The pressure at any point in the atrial cavities was in this way countered by an equal pressure from the weight of the surrounding fluid. The addition of further fluid would cause the intra-atrial pressure to rise above the pressure exerted by the fluid in the container. Therefore, the fluid level of the container was taken as the zero point in measuring atrial pressure.

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After the atria were completely filled in this fashion, measured amounts of saline were added to each atrium by a syringe. The atrial pressure was recorded optically with modified Gregg manometers after each volume increment was added.

#### RESULTS AND DISCUSSION

The elastic properties of the right and left atrium in relation to their volume were studied in 10 hearts. The amount of fluid needed to completely fill the atrial system without distention was measured in each case. The average volume of the right atrial system was 7.0 cc. with a range of 3.3 to 10 cc., while the left atrial system had an average volume of 3.4 cc. with a range of 1.3 to 4.8 cc. In all cases the right atrial system had a considerably greater volume than the left. This is a somewhat larger

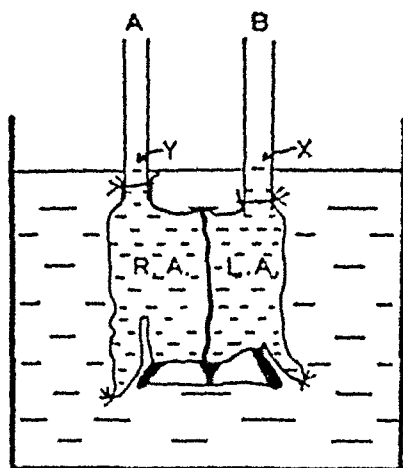


Fig. 1. METHOD OF DETERMINING filling volume of the atria by submerging under water. R. A., right atrium; L. A., left atrium. See text for further discussion.

difference than the one reported in human hearts by Hochrein and Eckardt (7). This initial volume is the capacity of the atrial system before it undergoes distention. When filled with any amount less than this initial volume it is a collapsible structure.

After the initial filling volume had been determined, the increments of additional fluid ( $dv$ ) were calculated on a percentage basis  $\left(\frac{dv}{V}\right)$ . Relative volume elasticity curves

were plotted by using  $\frac{dv}{V}$  as the ordinate, and the change in pressure ( $dp$ ) as the abscissa. In 7 out of 10 hearts studied the curves were similar. An example of the type of curves obtained from these 7 hearts is shown in figure 2 A. The curve of the right atrial system rises more steeply than the left and the two curves tend to diverge at higher volumes. This indicates that the pressure in the right atrium is less than in the left for the same percentage increase of volume. Thus, it must be inferred that the right atrial system is more distensible than the left.

In 3 cases the type of curve was different from the example presented. In one case the curves did not diverge but crossed after the volume was increased by 225 per cent. In this case the left atrial system became more distensible than the right beyond this point. In another of these atypical curves both right and left curves

were alike. In the third case the curves were reversed because the left atrial system was more distensible than the right over the entire range tested.

Within the normal range of atrial pressure (25-150 mm. of water) a linear relationship is apparent between volume and pressure. Above this range the relationship becomes nonlinear. Using the usual formula  $dv/dp \cdot V = Ev$ , where  $dp$  is the change in pressure,  $dv$  is the change in volume, and  $V$  is the initial volume, the volume

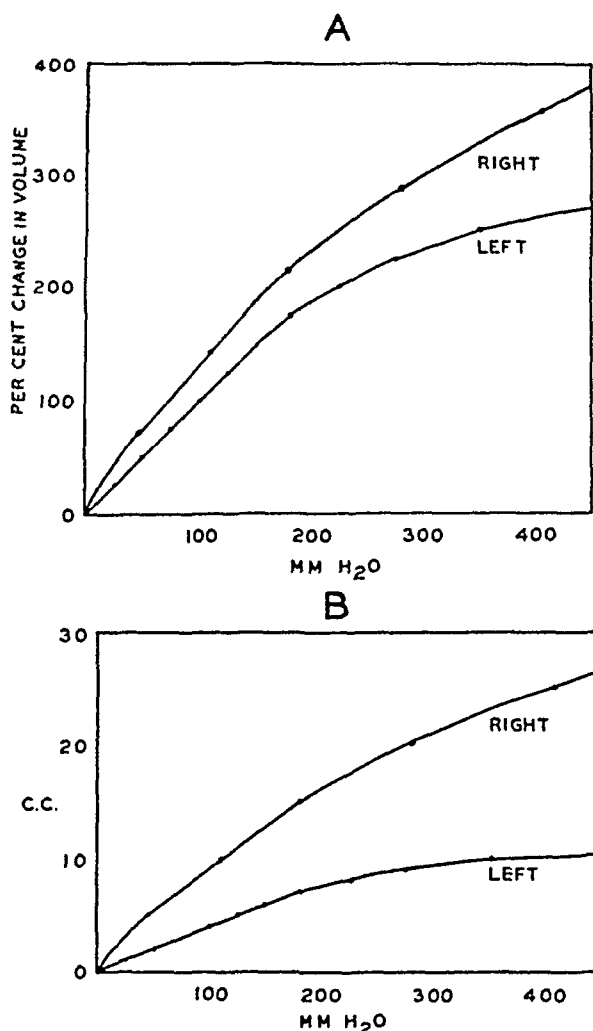


Fig. 2. VOLUME ELASTICITY CURVES for right and left atrial systems. A, relative curves; B, absolute curves.

elasticity coefficient ( $Ev$ ) of the right and left atrial systems can be calculated for the linear range of the curve. The  $Ev$  index for the right atrial curve shown in figure 1 is 92, while the left atrial system has an  $Ev$  index of 100. The average  $Ev$  index of the right atrial systems in this series is 78 with a scatter of 60 to 100. The average left atrial system  $Ev$  index is 89 with a scatter of 48 to 140.

While such relative elasticity curves are of value in comparing the physical characteristics of the walls of the right and left atrial systems, they are not adequate for hemodynamic studies of the intact atrial system.

Under stabilized conditions the flow through each side of the heart is the same,

thus during any phase of the cardiac cycle the volume change of the right and left atrium should be equal. In order to calculate the resulting pressure in each atrium from the volume elasticity curve, the curve must be adjusted for the difference in the initial volume. When increments of additional fluid are plotted on a percentage scale it will enlarge the left curve to twice the scale of the right since the right atrial initial volume is about twice that of the left. This may be adjusted simply by using  $dv$  as the abscissa and plotting the curve in the same manner as before. Such a procedure reduces the two plots to the same scale and tends to further separate the curves. The data from figure 2 A has been recalculated in this manner and are presented in figure 2 B.

All the experiments were re-plotted in this fashion and in every case for any given increment of volume the pressure was higher in the left than in the right atrial system. This is in general agreement with *in vivo* studies (1). The pressure differential between the right and left atria is a result of the difference in the volume of the two systems as well as the difference in their distensibility characteristics.

#### SUMMARY

Hearts were removed from dogs soon after death and the veins entering the atria were ligated. The A-V valves were sutured in the position of anatomical closure. The amount of fluid needed to completely fill the atria without distending them was measured. Measured amounts of additional fluid were added and the atrial pressure recorded with optical manometers after each increment of fluid was added.

The right atrial system as measured had an average initial filling volume twice that of the left atrial system.

Volume elasticity curves plotted for the right and left atrial systems show that for equal volume increments the right atrial system is more distensible than the left.

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# DYNAMICS OF EXPERIMENTAL ATRIAL SEPTAL DEFECTS<sup>1</sup>

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IT HAS been revealed in catheterization studies on humans with atrial septal defects (1, 2) and on dogs with experimentally produced defects (3) that left atrial pressure is greater than right in spite of apparently large interatrial communications. These observations, together with the demonstration that the oxygen saturation of right atrial blood is increased by contamination with arterialized blood from the left atrium (2), validate a long established clinical impression that a left to right shunt of blood occurs in such cases.

However, while the demonstration of a pressure differential between the atria provides a physical basis for the shunt of blood, two questions remain unanswered. First, how can such a pressure differential exist between the atria in the presence of a large communication, and second, what hemodynamic factors are responsible for maintaining this gradient?

Barger, Edwards, Parker, and Dry (4) have assumed on theoretical grounds that a large atrial septal defect should result in the equalization of right and left atrial pressure, presumably by conversion of the two atria into a common pressure chamber. The maintenance of a shunt of blood (and presumably the pressure differential?) then depends on the relative resistance to filling of the right and left ventricles. Taylor *et al.* (5) also came to much the same conclusion.

In an attempt to answer the second question, Uhley (6) has made the unlikely suggestion that the left to right shunt is due to gravitational factors resulting from the position of the heart in the thorax. Cournand *et al.* (1) have offered two explanations for this pressure gradient. First, that it may be maintained by the right atrium contracting slightly before the left, thereby increasing the initial tension of the left atrium by the transmission of pressure through the defect. Secondly, a more likely mechanism was suggested by the anatomic difference between the right and left atrial wall and the relative volumes of the two chambers. Thus, they postulated that the right atrium would be more distensible than the left. Opdyke and co-workers (7) also accounted for the pressure gradient between the atria in normal hearts as due to a difference in the elastic characteristics of the atrial wall and postulated that the pressure differential should persist after the production of a septal defect. This view is strengthened by the work of one of us, presented in the preceding paper (8), in which the right atrial system was shown to be more distensible than the left.

In view of the theoretical nature of the explanations for this pressure gradient so far presented, it seemed desirable to establish the basic hemodynamic factors involved in atrial septal defects. To this end defects were surgically produced in the atrial septum of dogs and the pressure relations studied. It is important that such experiments be done on an acute basis to avoid complicating compensatory develop-

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ments such as cardiac hypertrophy and change in blood volume, which are the result and not the cause of hemodynamic change.

#### METHODS

Average size mongrel dogs were anesthetized with intravenous sodium barbital (ca. 350 mg/kg.), or with 3 mg/kg. of morphine subcutaneously and 200 mg/kg. of sodium barbital intravenously. The chest was opened by a mid-sternal incision, and in most animals the right fifth rib was removed. The heart was suspended in a pericardial cradle which did not impede venous return. The surgical procedures were done with care to avoid undue blood loss and excess trauma. Mild but adequate respiration was maintained by means of alternating positive air pressure which was interrupted for brief periods when records were taken.

Aortic pressure pulses were secured by introducing a cannula through the right carotid artery to the arch of the aorta. The right external jugular vein was cannulated with a sound of as large a diameter as the vessel would permit. The slightly curved distal end was introduced into the right atrium. Left atrial pressure pulses were recorded from a cannula which entered the atrium through the tip of its appendage. All cannulae were fixed to avoid extraneous vibrations.

Pressure pulses were recorded with Gregg design optical manometers of adequate sensitivity and frequency. No parallax existed between the various recording beams. At the end of each record a short calibration was made with a standard pressure for each manometer in relation to its base line, and a complete calibration was done at the end of each experiment. The manometers used to record atrial pressure were calibrated in mm. water while the aortic manometer was calibrated in mm. Hg. In each experiment the zero level for all manometers was set at the level of the animal board. Therefore, all pressures are relative.

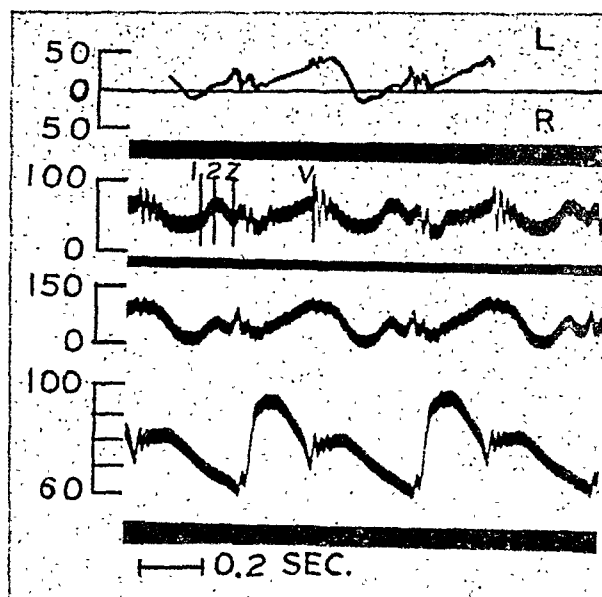
Atrial septal defects were produced by two methods. In early experiments a hemostat was introduced through the tip of the left atrial appendage and thrust through the septum. Opening the instrument and withdrawing it into the left atrium produced a slit or oval defect 5 to 8 mm. in its largest diameter. In later experiments the method of Blalock and Hanlon (9) was used to produce the defect under direct vision. Larger defects could be produced by this method. Following the production of the lesion by either method the animals were in good condition as indicated by normal contours of the aortic pressure pulse. Cardiac irregularities or other signs of cardiac failure were lacking. All lesions were examined and measured post mortem.

*Measurement of Atrial Pressures.* In view of the stand already taken by some of us (7) that mean atrial pressure is not an adequate measurement for hemodynamic studies, it seems appropriate to record our thesis in greater detail. It has been the practice in this laboratory when analyzing records to measure atrial pressure at 4 separate points on the pressure curve: beginning and peak of atrial systole, and beginning and end of ventricular systole. These points are shown in figure 1.

While these instantaneous pressures are all affected by venous return, they represent, in addition, the effects of various dynamic factors operating at different times during the cardiac cycle. Pressure recorded at the beginning of atrial systole (point 1) is in part a function of elastic properties of the combined atrial-ventricular-venous

system as well as the rate and amount of filling of the ventricle during diastasis. The pressure at the peak of atrial systole (point 2) is influenced by the muscular contraction of the atrial myocardium and the distensibility of the ventricle. While the pressure measured just before the beginning of ventricular systole (Z point) is chiefly dependent on the volume elastic characteristics of the common venous-atrial-ventricular cavity, pressure recorded just before the second heart sound (V point) is a function of the distensibility of the atrial-venous system, the length of ventricular systole and the rate of venous return.

Fig. 1. SIMULTANEOUSLY RECORDED right and left atrial pressure pulses with a constructed differential curve. From top down: constructed curve, right atrial, left atrial, and aortic pressure pulse. Letters referred to in text.



We regard the V point as the most significant measure of atrial pressure when dealing with interatrial dynamics: *a*) It has been shown that this is the only point that occurs synchronously in both right and left atrial pressure curves (7). *b*) This point is relatively free from impact artifacts resulting from the descent of the cardiac base during early ventricular ejection. *c*) At this point the A-V valves are closed so that a true intra-atrial pressure is recorded without any ventricular component. *d*) The atrial volume distensibility characteristic and the length of ventricular systole will vary only slightly in the course of an experiment and since there is no atrial out-flow during ventricular systole, changes in pressure at the V point reflect chiefly changes in venous return.

Our objection to using mean atrial pressure measurements in hemodynamic studies may be stated simply. Mean atrial pressure is a composite of all dynamic factors operating during one or more cardiac cycles. This is modified to an unknown degree by all artifacts inherent in or accidentally introduced in the process of recording. Significant dynamic events are therefore submerged and the influence of artifacts may profoundly affect the interpretation of results.

## RESULTS

Right and left atrial pressures were recorded from 12 dogs following the production of an atrial septal defect. Right atrial pressure measured at the V point varied

from 40 to 130 mm. with an average of 99.8 mm. of water, while left atrial pressure ranged from 104 to 195 mm. with an average of 149.7 mm. of water. Eleven of the 12 animals studied had a left to right inter-atrial pressure gradient. The differential pressure between the atria in these 11 experiments ranged from 13 to 130 mm. of water with an average of 49.9 mm. of water. There was only one experiment which did not have a left to right pressure differential. The loss of this gradient was due to a low left atrial pressure of 104 mm. water (16 mm. lower than any other in series), while the right atrial pressure of 104 mm. water was very near the average of the group.

The possibility existed that the pressure gradient might reverse during some phase of the cardiac cycle. In order to explore this possibility differential curves were constructed by subtracting the right atrial pressure pulse from the left by means of the coordirectograph described by Green. A typical record of right and left atrial pressure pulses and a constructed differential curve are presented in figure 1. By means of a constructed curve such as this the direction and magnitude of the pressure gradient between the atria is shown for each part of the cardiac cycle. The pressure gradient was found to persist from left to right during the entire cycle or for all but a very short period before or during atrial systole. In the experiment presented the reversal of the gradient occurred for 0.7 second just before atrial systole.

In order to determine if there is any change in the normal pressure gradient between the atria after the production of a shunt, atrial pressure pulses were recorded from the same animal both before and after production of the septal defect. The significant finding is that the V pressure differential fell from 90 mm. in the control to 70 mm. in the presence of the defect. This change in the pressure gradient resulted entirely from an increase of right atrial pressure.

If the two atria behave as a common chamber in the presence of a septal defect as suggested by Barger *et al.* (4), it may be expected that any increase in pressure in one atrium would be equally transmitted to the other. This can be tested by increasing right atrial pressure by a rapid infusion and recording the pressure changes in the right and left atria. It has been shown that in normal hearts when right atrial pressure is increased, left atrial pressure rises even more, thereby increasing the normal left to right pressure gradients (7). The effects of increasing venous return were observed in 12 dogs with atrial septal defects by giving rapid intravenous infusions of saline. The femoral vein was used in all cases and amounts varying from 75 to 405 cc. were given in 45 to 60 seconds.

These experiments can be divided into two groups. In the early animals of this series septal defects measuring 5 to 8 mm. were produced. These defects were considered to be the equivalent of a moderate size shunt. The later experiments had larger defects measuring 8 to 12 mm. in their largest dimension. These defects were considered to be large communications. Plots of typical experiments from each of these groups are presented in figure 2 as *A* and *B*. In each case 200 cc. of saline were given in 40 to 45 seconds.

Plot *A* represents an infusion into an animal with a moderate sized atrial septal defect. Right and left atrial pressure increased continuously during the infusion, left rising more than right. The pressure differential increased steadily from 26 mm. at

the start of the infusion to 85 mm. of water at the end. These results were consistent in 5 of the 7 animals in this group. In the other two experiments right atrial pressure increased more than left. In one of these the pressure gradient reversed during the infusion and in the other right atrial pressure continued higher than left throughout the experiment.

Plot B is representative of all infusions into the animals with larger inter-atrial defects. In this experiment both right and left atrial pressures increased at first, the pressure gradient between the atria increasing slightly during the first 50 cc. of the

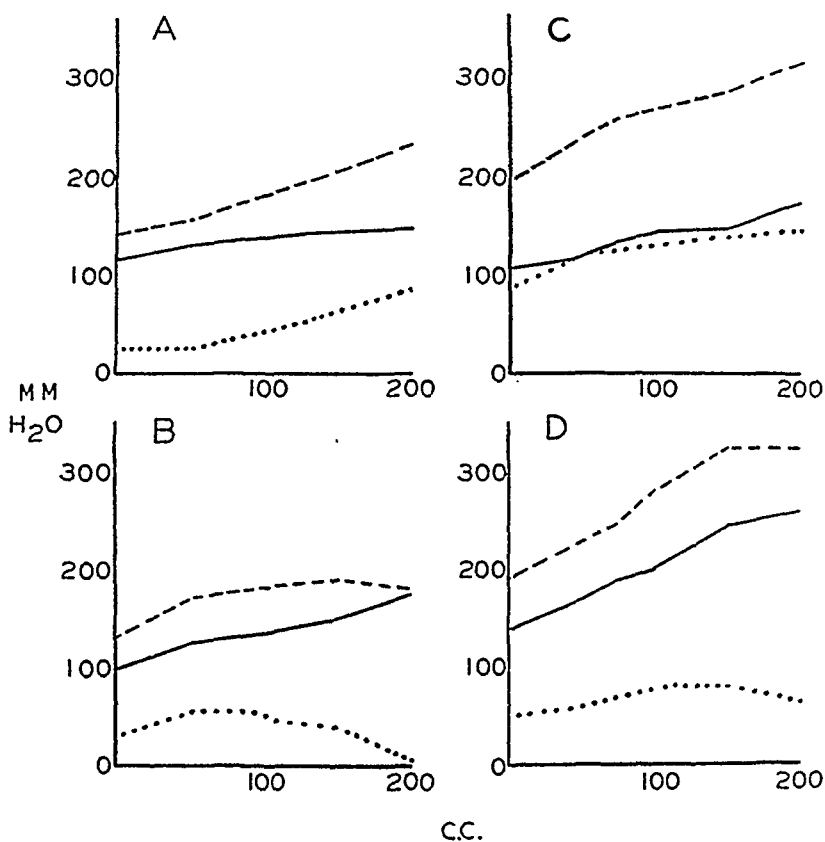


Fig. 2. PLOTS OF RIGHT AND LEFT atrial pressure at V point during an intravenous infusion of 200 cc. of saline in 45 seconds. Solid lines, right atrial pressure. Broken line, left atrial pressure. Dotted line, differential pressure between atria. Discussion in text.

infusion due to the greater increase of left atrial pressure. During the next 100 cc. the pressure differential began to fall slightly and then decreased markedly during the last part of the infusion. This decrease was due to a fall of left atrial pressure.

In order to compare the results of these infusions into hearts with septal defects with expected results in normal hearts, a series of similar infusions was done in normal animals. The results were consistent in 4 experiments. A plot of a representative experiment is shown in figure 2 C. Both right and left atrial pressures increased, left more than right, throughout the infusion. This is in agreement with previously reported results (7).

As a further comparison with the normal, a large atrial septal defect was made in the same animal which is presented as the control in figure 2 C. This experiment

(fig. 2 *D*) is similar to that plotted in figure 2 *B*, except that after a considerable amount of fluid had been infused the gradient between the atria decreased because left atrial pressure leveled off rather than decreased.

Another method of determining if the two atria act as a common chamber in the presence of an atrial septal defect is to study the atrial pressures obtained under static conditions. When ventricular asystole is produced in a normal heart by stimulation of the peripheral end of the left vagus nerve, right atrial pressure continues to increase due to the persistence of venous return, as shown by the significant sections of a continuous record reproduced in figure 3 *A*. Left atrial pressure increases for 2 to 3

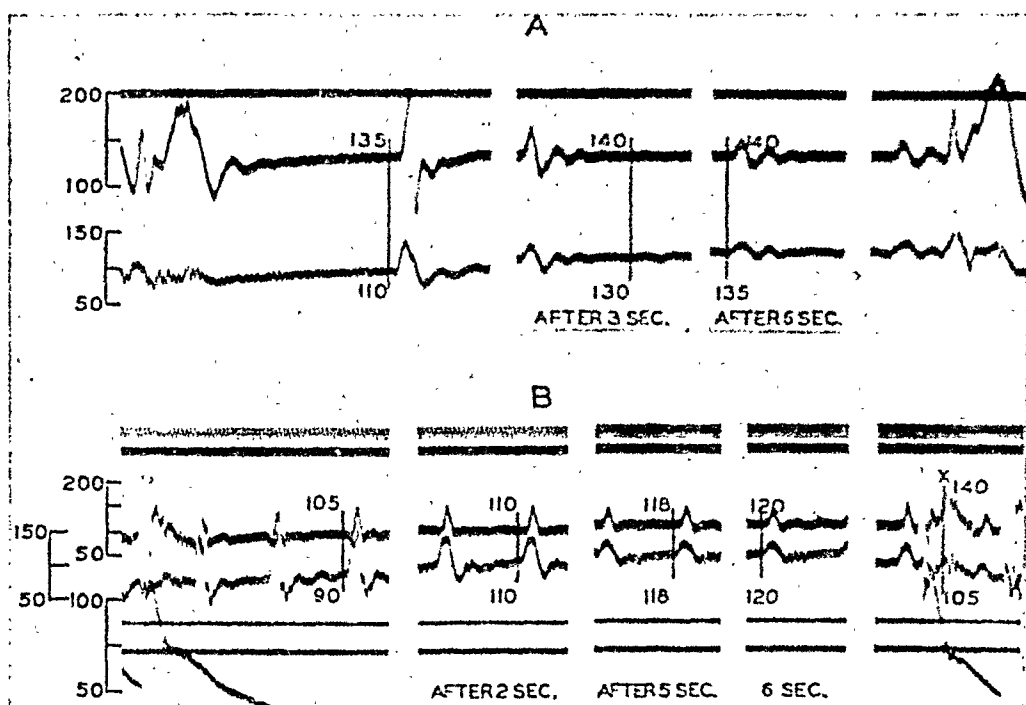


Fig. 3. SECTIONS OF CONTINUOUS RECORD of left atria pressure (*upper*) and right atrial pressure (*lower*) during and after ventricular asystole. *A*, normal heart; *B*, heart with atrial septal defect. See text for discussion.

seconds (2 cycles), then remains constant for the duration of the ventricular standstill. The elastic recoil of the lungs returns a small volume of blood to the left atrium, but after a short interval this ceases and left atrial pressure does not increase further.

Figure 3 *B* shows sections of a continuous record from a similar experiment performed on a heart *with a large atrial septal defect*. In contrast to the normal heart, right and left atrial pressures equalize within 2 seconds (6 cycles) and then rise together for the remaining 5 seconds of ventricular standstill. Following the first ventricular beat, a pressure differential of 35 mm. of water is again present between the atria (see point X on record).

#### INTERPRETATION

The above experiments indicate that the atria act as a common cavity in the presence of an atrial septal defect under static conditions. This is shown by the rapid

equalization of pressure when ventricular asystole is produced. However, when ventricular action is restored there is an immediate reestablishment of the pressure gradient. This shows that the pressure differential is produced by dynamic events.

An analysis of the factors involved in maintaining this dynamic gradient can be based on the following factors: The pressure in the atria of the heart during atrial diastole is dependent on the inflow, the outflow, and the distensibility of their walls. The demonstration that the right atrium is more distensible than the left (8) offers a logical explanation for the maintenance of this pressure gradient between the atria.

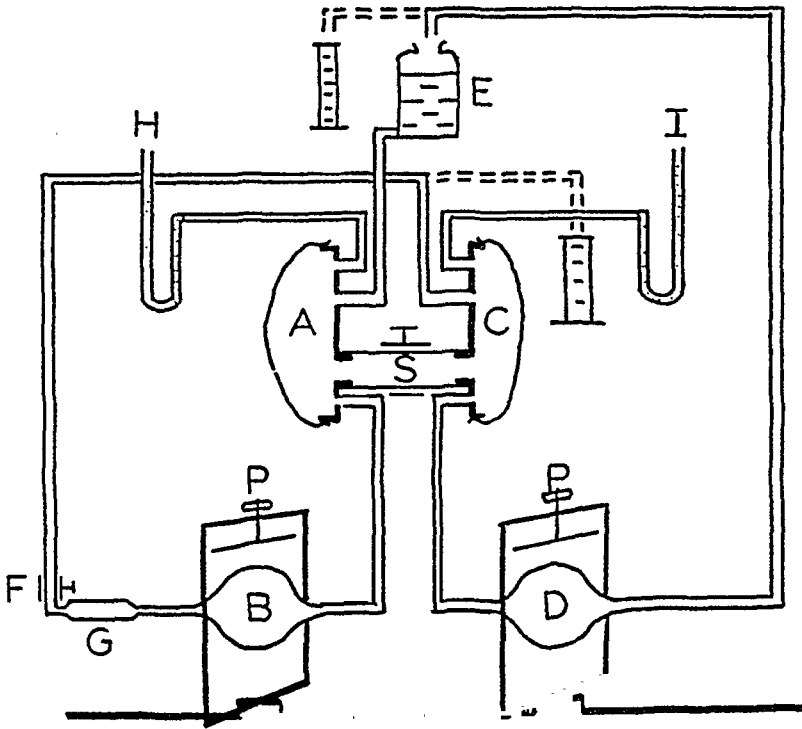


Fig. 4. DIAGRAM OF PHYSICAL MODEL used to illustrate the dynamics of atrial septal defects. Letters referred to in text.

The inflow into the two atria must be equal if the circulation is to be stabilized. In the presence of a septal defect the inflow into the right atrium consists of the venous return plus the flow through the shunt from the left atrium. This same volume of blood is ejected by the right ventricle and returns to the left atrium through the pulmonary circuit. Thus right atrial inflow equals left atrial inflow. The outflow from the two atria is also equal; right atrial outflow equals right ventricular output, while left atrial outflow equals the flow through the shunt plus left ventricular output. Since the inflow of the left atrium is equal to the outflow of the right ventricle, it must also equal right atrial outflow. Thus right atrial outflow must equal left atrial outflow.

The left atrium is a less distensible structure than the right and has a smaller volume (8). Since the flow through both atria is equal, the chamber having the less distensible walls and the smaller volume will develop the higher pressure. For this reason left atrial pressure is higher than right in the normal heart (7) and in hearts with atrial septal defects.

The dynamic factors concerned in maintaining this higher left atrial pressure

can be illustrated by means of a physical model such as is diagrammatized in figure 4. Two brass plates, A and C, are covered with rubber dam of different distensibilities. Their volume elasticity curves are similar to those of the two atria (8). A shunt S between these atria can be closed by a clamp. A reservoir E is connected to the right atrium. The two atria are connected by a tube in which is a rubber bulb B fitted with appropriate valves. The left atria is connected to a similar bulb and valve D which empties into the reservoir E. The rubber bulbs are synchronously compressed by adjustable motor driven plungers P and P'.

The entire system is filled with water and the shunt between the atria is closed. The motor driven plungers that compress the bulbs are driven at 40 beats per minute. A resistance F, which is placed ahead of a distensible section of tubing G, is adjusted to provide a steady flow of fluid into the left atrium C. It is important that the two plungers be adjusted so that each bulb pumps the same volume; when this is done

TABLE 1

	VENTRICULAR OUTPUT IN	ATRIAL PRESSURE
	cc/min.	mm. water
Normal		
Right.....	70	40
Left.....	70	155
Lesion		
Right.....		49
Left.....	44	50
Compensated lesion		
Right.....	164	55
Left.....	70	155
Flow through shunt = 94 cc. per minute.		

the level in the reservoir remains constant. Atrial pressure can be measured with sufficient accuracy in this case by water manometers H and I. Left ventricular output is determined by permitting the inflow to the reservoir E to flow into a graduate. Right ventricular output is measured by disconnecting the inflow into the left atria and measuring it in a graduate.

Data from a typical experiment with this model are shown in table 1. With the shunt closed a pressure differential of 115 mm. of water is present between the atria. When the shunt is opened the pressures equalize, the right atrial pressure rising slightly above the control. Right ventricular output is then increased by adjusting the thrust of the plunger that compresses the bulb B. When left atrial pressure has risen to control levels a pressure differential between the atria of 100 mm. of water is again maintained, the right atrial pressure increasing slightly. Left ventricular output is back to control levels while right ventricular output is more than doubled. The difference between right and left ventricular output must equal the amount passing through the shunt, as the level in the reservoir E remains constant. The conditions with the shunt open are the same as those demonstrated in humans with atrial septal defects, i.e., left atrial pressure higher than right and right ventricular output nearly twice that of the left ventricle (2).

To demonstrate that a difference in the distensibilities of the two atria is required to produce this left to right pressure gradient, the physical model is modified as follows: The rubber membrane A which covers the right atrium is removed and a rubber membrane with the same elastic characteristics as that covering the left atrium is substituted. Thus, both atria have the same volume elastic characteristics. When the model is operated under the same conditions as before, a pressure differential between the atria does not occur and consequently there is no flow through the shunt.

Evidence derived from *in vivo* observations, static volume elasticity measurements on the atria, and experiments with the physical model prove conclusively that the pressure differential existing between the left and right atria depends on the lesser distensibility of the left atrium and the increase in right ventricular output. The role of ventricular filling in maintaining this pressure differential must be minimal since the differential pressure is greatest at the V point when the ventricles are shut off from the atria by the closed A-V valves and the differential pressure is markedly reduced during the period of ventricular filling. The decrease of the inter-atrial pressure differential during ventricular filling is due to the fact that the volume-elasticity characteristics of the right and left atrio-ventricular cavities are more nearly similar (7). However, the importance of the right ventricle's ability to pump an increased volume of blood is obvious.

The effect of increasing venous return on right and left atrial pressures in the presence of an inter-atrial shunt is explainable on the basis of this dynamic mechanism. In order to maintain left ventricular output in the presence of an atrial septal defect, the right ventricle must eject not only the blood that is normally returned to the heart but also any which passes through the shunt from the left atria. When venous return is increased, as by a rapid intravenous infusion, the compensatory mechanisms of the right heart must operate to their utmost capacity in order to keep the circulation in equilibrium. When the atrial septal defect is of small or moderate size the right heart is able to fully compensate for the rapid infusion of 200 cc. of saline as is shown by the rise of right and left atrial pressure during the entire infusion, much as in the normal heart (plot A and C, fig. 2). However, in the presence of a larger shunt, the increased flow from the left heart plus that which comes from the periphery must already have placed a burden on the compensatory mechanism of the right heart. In the presence of a great increase in venous return, right ventricular output at first increases but the degree that its output is augmented may not continue proportional to the increase in right atrial pressure. This is due to the right ventricle becoming less efficient at the higher filling pressure (10). Under stabilized conditions left venous return is dependent upon right ventricular output, thus left atrial pressure will rise with increased right heart output. When the right heart output fails to keep up with the increased flow through the shunt, left atrial pressure will plateau (fig. 2 D) or may even fall (fig. 2 B).

#### SUMMARY

Atrial septal defects were surgically produced in open chest dogs and right and left atrial pressure pulses were simultaneously recorded by calibrated optical manometers. Atrial pressure was measured just before the second heart sound, the V point.

The use of other points on the atrial pressure pulse as a measure of atrial pressure is discussed.

In 11 out of 12 experiments left atrial pressure was higher than right at the V point and the left to right pressure gradient persisted during the entire cardiac cycle, or for all but a short period before or during atrial systole.

Increasing venous return by rapid saline infusion elevates both right and left atrial pressure in the presence of atrial septal defects, but left atrial pressure rises more than right. However, in the presence of a large defect the pressures tend to equalize after considerable fluid has been given.

The suggestion that in the presence of atrial septal defects the atria would act as a common cavity was tested. When ventricular action was stopped by vagal stimulation, right and left atrial pressure equalized within 2 seconds and then rose together for the duration of ventricular systole. Following the first ventricular beat, the left to right pressure differential was again present. Thus the inter-atrial pressure gradient is shown to be produced by dynamic events.

Evidence derived from *in vivo* observations, atrial volume elasticity measurements, and experiments with a physical model prove that the left to right pressure gradient between the atria in hearts with atrial septal defects depends on the lesser distensibility of the left atrium and the increase in right ventricular output.

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# METABOLISM OF CARDIAC MUSCLE: UTILIZATION OF C<sup>14</sup> LABELLED PYRUVATE AND ACETATE BY RAT HEART SLICES<sup>1</sup>

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THE effect of addition of various metabolites on the respiration of surviving tissue slices has afforded some, but incomplete, information on the ability of the tissue to utilize a particular substrate. Chemical measurement of the rate of disappearance of substrate added to surviving tissues affords a more quantitative index of the ability of the tissue concerned to utilize the metabolite but does not indicate the end products of the chemical transformations that may occur. With the use of metabolites labelled with C<sup>14</sup> it is possible to determine the amount of substrate which is oxidized to CO<sub>2</sub> as well as the incorporation of the tracer into certain tissue components.

It was of interest to determine the ability of rat cardiac muscle slices to utilize pyruvate labelled with C<sup>14</sup> in the alpha position and acetate labelled with C<sup>14</sup> in the carboxyl position. Oxidation of the substrate to CO<sub>2</sub> was determined by measuring the radioactivity in the respiratory CO<sub>2</sub>. The amount of radioactivity in the fat and protein fractions of the tissue after incubation was also determined.

## METHODS

Wistar-strain rats, varying in weight from 200 to 400 grams and in age from 3 to 6 months, were used. They were fed Purina Dog Chow *ad libitum* and were not fasted before death. The animals were killed by a sharp blow on the head, and the heart was excised as rapidly as possible and placed in an aliquot of iced, phosphate buffered medium whose ionic composition was as follows:

CATION	CONCENTRATION <i>mEq/l.</i>	ANION	CONCENTRATION <i>mEq/l.</i>
Na	150	Cl	142
K	4	HPO <sub>4</sub> }	15
Ca	2	H <sub>2</sub> PO <sub>4</sub> }	
Mg	1		

The pH was 7.4. Eleven mM per liter of glucose was added in all experiments except when the utilization of other substrates was being studied.

Heart slices approximately 0.5 mm. in thickness were prepared by hand using a razor blade held in a hemostat as a cutting tool and a glass slide as a template. A

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metal block, chilled in ice, was used as a base. The razor blade and glass slide were dipped frequently into chilled saline medium so that the cardiac tissue and the cutting implements were kept chilled during the period of slicing. The whole ventricles were placed on their side on the metal block, and slices were made along the longitudinal axis of the heart, cutting through the two chambers and septum as they were encountered. As each slice was prepared, it was placed in an aliquot of well oxygenated incubation medium at room temperature. The approximate thickness of tissue slices was determined by dividing the wet weight of the tissue slice by the surface area without correction for specific gravity of the tissue.

Respiration was measured at 37° C. by the direct method of Warburg. Each vessel contained 3.0 ml. of the incubation medium in the outer compartment and 0.2 ml. of 10 per cent KOH plus filter paper in the center cup. One tissue slice, varying in (dry) weight from 10 to 20 mg., was placed in each vessel. The vessels were equilibrated with 100 per cent oxygen for 5 minutes while being shaken in the constant temperature bath at the rate of 120 oscillations per minute. Readings were started about 30 minutes after the animals had been killed, and subsequent readings were made at 10 to 15 minute intervals for periods of 1 to 3 hours. The tissue was removed at the end of the experiment and the dry weight obtained. Determinations were made in duplicate or triplicate. Respiration rate curves were plotted and the best linear relationship obtained from the observed points. The results usually checked within 15 per cent and are expressed as  $Q_{O_2}$  (mm.<sup>3</sup> of O<sub>2</sub>/mg. of dry weight of tissue/hr.).

Unlabelled sodium pyruvate used in these experiments was prepared by redistillation of pyruvic acid according to the method of Lipschitz, Potter and Elvehjem (1), neutralization with NaHCO<sub>3</sub> and precipitation in alcohol. Pyruvate was determined by the direct method of Friedemann and Haugen (2). Lactate was determined by the method of Barker and Summerson (3). Ten per cent trichloroacetic acid filtrates of the incubation medium were prepared for the determination of pyruvate and lactate. Pyruvate utilization is expressed in terms of  $-Q$  pyruvate (microliters of pyruvic acid removed/mg. dry weight of tissue/hr.; 1 microliter = 0.0446 micromols.) and lactate formation in terms of  $Q$  lactate (microliters of lactic acid formed/mg. of dry tissue/hr.).

Potassium pyruvate labelled with C<sup>14</sup> in the alpha carbon position and sodium acetate labelled with C<sup>14</sup> in the carboxyl position were prepared by Dr. Yale Topper. The radioactivity of these compounds was determined as BaCO<sub>3</sub> after combustion of a sample to CO<sub>2</sub>, collection in alkali and precipitation with Ba(OH)<sub>2</sub>. The potassium pyruvate had an activity of 4000 counts per minute per milligram when referred to an arbitrary standard of BaC<sup>14</sup>O<sub>3</sub>. The sodium acetate had an activity of 10,800 counts per minute per milligram when referred to the same standard.

Oxidation of these substrates by cardiac muscle slices was determined by the percentage of the total activity placed in the incubating medium that was recovered in the respiratory CO<sub>2</sub>. This calculation involves the assumption that for each labelled carbon atom appearing in the respiratory CO<sub>2</sub>, two unlabelled carbon atoms from pyruvate and one unlabelled carbon atom from acetate also appear in the respiratory CO<sub>2</sub>. Two tenths ml. of 1 N H<sub>2</sub>SO<sub>4</sub> was placed in the side arm of the Warburg vessel

and was mixed with the incubating medium at the end of the experiment to release any  $\text{CO}_2$  that was retained in the buffered solution. The alkali in the center well and on the filter paper was transferred quantitatively to a 15 ml. centrifuge tube with 0.5N NaOH. The carbonate was precipitated as the barium salt by the addition of  $\text{Ba}(\text{OH})_2$ . The  $\text{BaCO}_3$  precipitate was centrifuged and washed once with an aliquot of 60 per cent alcohol. After centrifugation the  $\text{BaCO}_3$  was transferred to a stainless steel disc for counting.

Total fat and protein in the tissue was prepared for counting in the following manner. The tissue was removed from the Warburg vessel, rinsed briefly in distilled water, placed in a small glass dish and dried overnight in an oven at  $110^\circ\text{C}$ . The dry weight was obtained. The tissue was ground with a mortar and pestle in 10 cc. of 10 per cent trichloroacetic acid and transferred to a 15 cc. centrifuge tube. After centrifugation the precipitate was washed three times with 10 ml. aliquots of trichloroacetic acid. Ten ml. of alcohol-ether were added and the precipitate was dispersed with a stirring rod and allowed to stand overnight. The alcohol-ether mixture consisted of 3 parts ethyl alcohol and 1 part ethyl ether. On the following day the precipitate was extracted three times with 5 ml. aliquots of alcohol-ether, the mixture being brought to a boil during each extraction. The protein precipitate was transferred to a stainless steel disc and dried. The alcohol-ether extracts were evaporated to dryness and the fatty residue transferred to a stainless steel disc for counting.

The four washings of the ground tissue with trichloroacetic acid appear to be adequate to remove unchanged labelled pyruvate and acetate, since experiments in which the tissue and incubating medium were acidified to  $\text{pH}$  1 before adding the labelled substrates yielded no measurable counts in the fat and protein fractions of the tissue. Self absorption corrections for the fat and protein samples were made using the same factors as for  $\text{BaCO}_3$ .

## RESULTS

### *Factors Affecting Rate of Respiration of Cardiac Muscle Slices*

Preliminary observations on the rate of respiration of normal rat cardiac muscle slices yielded considerable variation in the  $Q_{\text{O}_2}$  values from animal to animal. Data in the literature (4-8) revealed variations in the  $Q_{\text{O}_2}$  values for rat heart slices from 4.0 to 10.0 under approximately similar conditions. Consideration was therefore given to possible variable factors in the preparation of the tissue for respiration measurements as well as to the effect of certain changes in the ionic composition of the suspending medium.

*Effect of slice thickness.* Fuhrman and Field (9) have demonstrated that the thickness of liver slices for optimum respiration is between 0.5 and 0.6 mm., and that lower rates of oxygen consumption are obtained when the slices are thinner or thicker. This tissue thickness producing optimum respiration corresponds very well with the theoretical limiting thickness for adequate diffusion of oxygen as calculated by the Warburg (10) formula. Presumably, the lower respiration of thin slices is due to a greater proportion of damaged cells, and of thick slices to an inadequate supply of oxygen.

Table 1 shows the results of experiments to determine the effect of tissue slice thickness on the rate of oxygen consumption of cardiac tissue slices. The tissue thickness for optimum respiration is approximately the same as that determined by Fuhrman and Field for liver slices. It is apparent from the data that the slice thickness is an important factor in determining the rate of respiration of heart slices. The optimum thickness for heart slices is considerably greater than that usually recommended for tissue slice work (11).

*Effect of anoxia.* Bernheim and Bernheim (4) have shown that semi-anaerobic incubation of the whole rat heart *in vitro* at 37° C. for periods as short as 5 minutes produces considerable depression in the rate of oxygen consumption of cardiac tissue slices. A similar depression in the respiration of cardiac muscle has been produced by anoxia *in vivo* by Fuhrman and Field (8) using decompression to produce anoxia, and by Burdette and Wilhelmi (6) using hemorrhagic shock. Lowering the oxygen tension with which cardiac tissue slices are equilibrated *in vitro* produces a marked lowering of the rate of oxygen consumption (12), and subsequent recovery of such slices upon increasing the oxygen tension is much less than is the case with liver slices.

TABLE 1. EFFECT OF SLICE THICKNESS ON O<sub>2</sub> CONSUMPTION OF RAT VENTRICLE

NO. OF ANIMALS		SLICE THICKNESS IN MM.		
		.25-.35	.5-.6	.8-1.0
7	-O <sub>2</sub> <sup>1</sup>	7.2±1.1 <sup>2</sup>	12.8±0.8	10.2±0.3

<sup>1</sup> Cu. mm. of O<sub>2</sub> consumed/mg. dry wt. of tissue/hr.

<sup>2</sup> Mean and standard error of the mean

An experiment was performed in which the heart was rapidly removed from the animal and placed in chilled incubating medium. Slices were cut as rapidly as possible and placed in an aliquot of incubating medium at room temperature with oxygen bubbling through the medium. Control slices were taken immediately for respiration measurements. The oxygen supply was then shut off and other slices taken at varying intervals thereafter for respiration measurements. The results are shown in table 2. It is apparent that brief periods of anoxia produce an irreversible change in heart muscle so that a permanent lowering of the rate of respiration occurs. Since anoxia is inevitable during the preparation of tissue slices, we have attempted to minimize this effect by chilling the tissue to 5° C. during the preparation of the slices.

*Effect of changes in ionic pattern of the incubating medium.* The omission of Ca<sup>++</sup> alone, or of the three cations, Ca<sup>++</sup>, Mg<sup>++</sup>, and K<sup>+</sup>, from the incubation medium had no significant effect on the rate of respiration of ventricle slices.

The effect of varying the proportion of Na<sup>+</sup> and K<sup>+</sup> in the incubation medium is shown in table 3. The proportions of these two cations were varied in such a way that the total concentration of the two was always the same. It will be noted that up to a concentration of 77 mM per liter of K<sup>+</sup>, there is little or no effect upon the rate of respiration of ventricle slices, whereas, above this concentration, a marked depression of respiration occurs. This *in vitro* effect of potassium ion on the respiration of cardiac muscle does not appear to be correlated with the effect of potassium on the contractility of the intact heart, since this latter effect occurs with potassium concentrations of approximately 10 to 20 mM per liter.

Table 4 presents the results of varying the total phosphate concentration in the incubation medium. The omission of phosphate ion from the medium, with or without the addition of a substitute buffer, results in a depression of oxygen consumption. Adding phosphate ion in increasing increments results in a stimulation of respiration with a maximum effect being produced at a concentration of 10 to 20 mM per liter. The use of isotonic phosphate buffer alone as the suspending medium results in maximum stimulation of respiration during the first hour, but there is a marked falling off in the rate of respiration during the second and third hours.

Lowering the  $pH$  of the incubation medium from 7.4 to 6.8 without changing the total concentration of phosphate ions produced a 25 per cent depression of the rate of respiration of ventricle slices. Raising the  $pH$  of the incubation medium to 7.9 resulted in no significant change in the rate of respiration.

*Effect of addition of various substrates.* The effect of addition of several substrates on the rate of respiration of cardiac muscle has been reported by several investigators. Smyth (13) has shown that sheep heart mince utilizes pyruvate, succinate, fumarate, citrate and  $\alpha$  ketoglutarate and that malonate inhibits the utilization of pyruvate, suggesting that the Krebs tricarboxylic acid cycle is active in heart muscle.

TABLE 2. EFFECT OF ANOXIA ON RESPIRATION OF HEART SLICES

PERIOD OF ANOXIA min.	$-Q_{O_2}$	% CHANGE
0	11.7	
5	8.8	-25
10	7.1	-40
20	6.3	-47

Bernheim and Bernheim (4) observed an increased rate of respiration of rat cardiac muscle upon the addition of succinate, actate, and pyruvate, but failed to obtain any effect with glucose, glucose-1-phosphate, glucose-6-phosphate, mannose, adenylic acid, glutamate, alanine, acetate, tyramine and fumarate. Burdette and Wilhelmi (6) found a depression of respiration with glucose and a stimulation with pyruvate. Angerer and Gonzales (7) found a depression of respiration with glucose and a stimulation with succinate and pyruvate.

The effect of addition of several substrates on the rate of respiration of rat cardiac slices is summarized in table 5. In all cases, the substrate was added at the beginning of incubation of the tissue slices. When tissue slices are incubated for 20 to 60 minutes before the addition of substrate, the response to the addition of substrate is often less than when it is added at the beginning of incubation. This may be a factor in accounting for some of the differing effects obtained by several investigators. The data suggest but do not prove that cardiac tissue slices can utilize added glucose, lactate, pyruvate, acetate, succinate, fumarate, and citrate.

*Effect of duration of incubation.* The rate of respiration of cardiac slices in the absence of added substrate shows a slight but progressive decrease with time. In the presence of added substrates which stimulate the respiration of cardiac muscle, the rate of oxygen consumption continues in almost linear fashion for the two-hour period of observation. Typical respiration plots are presented in figure 1.

Despite the maintenance of respiration for long periods in a medium to which glucose was added, histologic examination of the tissue slices reveals a progressive

deterioration of anatomical structure with time. Tissue slices were placed in Zenker's fluid. After paraffin imbedding, sections were cut at right angles to the surface of the slice and stained with eosin-methylene blue. Freshly prepared tissue slices placed immediately into Zenker's fluid showed normal histological structure except for irregular basophilia in the cytoplasm of the peripheral muscle cells involving the outer 3 to 4 layers of cells. This basophilia appeared in all sections prepared from tissue slices and is considered to be related to injury produced in slicing. Sections examined during the first hour of incubation showed no other striking cytologic changes. After one hour of incubation, eosinophilic granules appeared in the cytoplasm of some of the fibers and the myofibrils appeared to have swelled. There was also a loss in the cross striation of the muscle fibers stained with phosphotungstic acid-hematoxylin.

TABLE 3. EFFECT OF CHANGES IN THE NA AND K ION CONCENTRATIONS IN THE INCUBATION MEDIUM ON THE RESPIRATION OF RAT VENTRICLE:

NO. OF OBSERVATIONS	K <sup>+</sup> mEq/l.	Na <sup>+</sup> mEq/l.	-Q <sub>O<sub>2</sub></sub>	NO. OF OBSERVATIONS	K <sup>+</sup> mEq/l.	Na <sup>+</sup> mEq/l.	-Q <sub>O<sub>2</sub></sub>
7	4	136	7.6	5	65	75	8.5
1	17	123	6.9	3	77	63	8.3
1	25	115	7.6	1	93	47	3.6
1	33	107	7.1	1	112	28	2.7
1	41	99	8.3	5	136	4	3.3
1	53	87	8.2	1	140	0	1.5

TABLE 4. EFFECT OF VARYING THE PHOSPHATE CONCENTRATION IN THE INCUBATION MEDIUM ON THE RESPIRATION OF VENTRICLE SLICES

NO. OF OBSERVATIONS	P mm/l.	-Q <sub>O<sub>2</sub></sub>	NO. OF OBSERVATIONS	P mm/l.	-Q <sub>O<sub>2</sub></sub>
9	0	5.4	1	15.0	11.6
2	2.5	5.8	2	20.0	12.0
10	5.0	7.5	1	30.0	7.3
5	7.5	8.0	1	40.0	7.4
2	10.0	9.0	1	100.0	10.2

These changes increased with time of incubation, so that after 3 hours normal striated fibers were difficult to find.

Similar tissue slices were fixed in absolute alcohol-picric acid-formalin mixture and stained for glycogen by Bauer-Feulgen method. Glycogen was identified by its hydrolysis with saliva. Examination of these sections revealed that glycogen had completely disappeared from the central fibers of the slice within 5 minutes of their preparation. Glycogen was found to be present in the muscle fibers at the periphery of the slice after 2 to 4 hours of incubation. The rapid loss of glycogen from the center of the slice may be related to the lower oxygen tension in this area.

#### *Utilization of C<sup>14</sup> Labelled Pyruvate*

Preliminary experiments using unlabelled pyruvate showed that concentrations of 2.5 to 5.0 mM/l. of pyruvate yield optimum stimulation of respiration of cardiac slices. Chemical determination of the rate of pyruvate disappearance showed that relatively large amounts of pyruvate disappear during the first few minutes of incubation and that thereafter the rate of pyruvate disappearance is approximately

linear. Coincident with the initial rapid disappearance of pyruvate is the appearance of an increased amount of lactate which then remains approximately constant for the

TABLE 5. EFFECT OF ADDITION OF SUBSTRATES ON RATE OF RESPIRATION OF RAT VENTRICLE

NO. OF ANIMALS	SUBSTRATE	CONCENTRATION	INCREASE IN: O <sub>2</sub> CONSUMPTION
		mm/l.	
12	L Glucose	11	28
4	DL Lactate	5 to 60	56
14	Pyruvate	5	83
9	Acetate	5 to 20	55
1	Succinate	20	350
2	Fumarate	30	42
1	DL Citrate	40	25

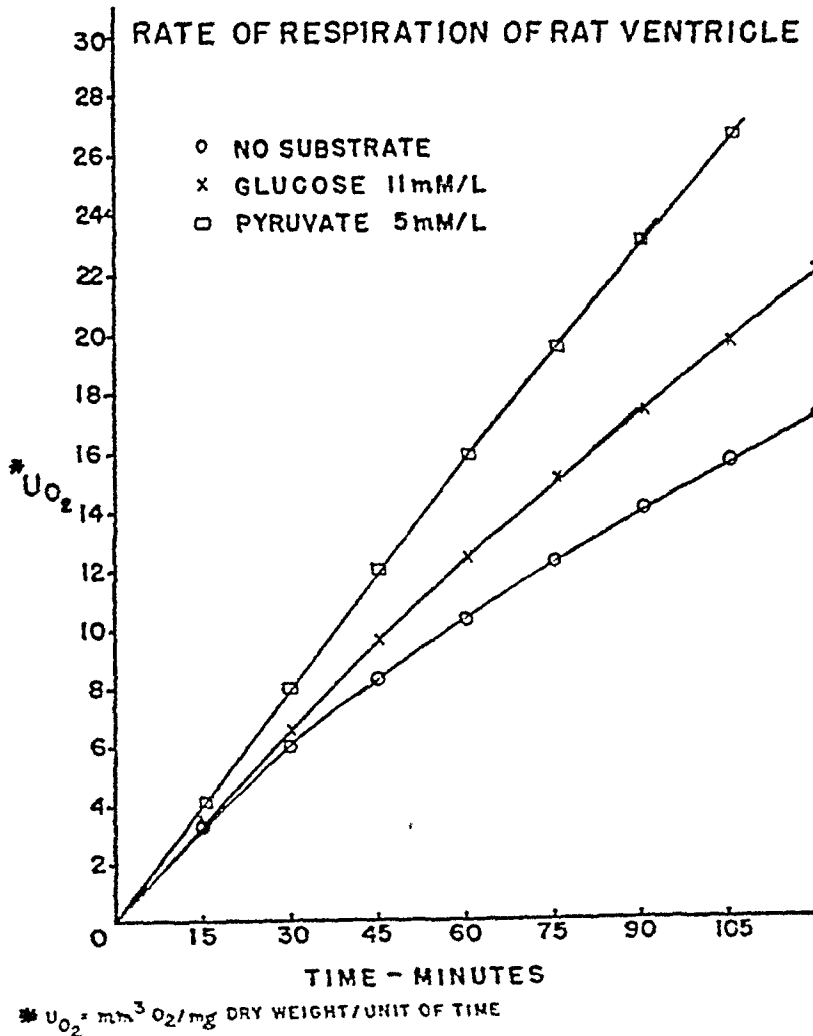


Fig. 1

rest of the experiment. These results indicate that a fraction of the pyruvate added is rapidly reduced to lactate.

The total amount of pyruvate disappearing ( $-Q$  pyruvate) has been corrected for the quantity of pyruvate which is reduced to lactate in the following manner.

The value for  $Q$  lactate obtained in the absence of added substrate is subtracted from the value for  $Q$  lactate obtained in the presence of added pyruvate to obtain the increment of pyruvate converted to lactate. This quantity is then subtracted from the total  $-Q$  pyruvate to obtain the net  $-Q$  pyruvate, which is an approximation of the number of microliters of pyruvate converted to non-lactate products.

TABLE 6. UTILIZATION OF PYRUVATE BY NORMAL RAT VENTRICLE

RAT NO.	$-Q_{O_2}$	$Q_{LACTATE}$	$-Q_{O_2}$	$-Q_{PYRUVATE}$	$Q_{LACTATE}$	NET $-Q_{PYRUVATE}$
	<i>No added substrate</i>		<i>Pyruvate—5 mm/l.</i>			
1	5.4	0.77	11.0	6.95	2.28	5.47
2	8.0	0.49	16.1	6.46	2.70	4.25
3	9.0	1.08	12.4	4.83	2.64	3.27
4	8.1	0.71	13.2	5.99	2.41	4.29
5	8.6	0.52	15.8	6.40	2.95	3.95
6	8.7	0.90	15.6	7.03	3.28	4.65
7	10.8	0.50	18.0	8.60	2.48	6.62
8	11.0	0.76	20.5	9.33	3.08	7.01
9	10.8	0.47	16.1	9.39	3.94	5.92
Av.	8.9	0.69	15.4	7.22	2.86	5.05±0.43

TABLE 7. UTILIZATION OF  $C^{14}$  LABELLED PYRUVATE IN RAT VENTRICLE SLICES

RAT NO.	$-Q_{O_2}$	$Q_{LACTATE}$	$-Q_{O_2}$	NET $-Q_{PYRU-}$ VATE	$Q_{LACTATE}$	NET $-Q_{PYRU-}$ VATE	* $CO_2$ $-Q_{PYRU-}$ VATE	*FAT $-Q_{PYRU-}$ VATE	*PROTEIN $-Q_{PYRU-}$ VATE
	<i>No Substrate</i>		<i>Pyruvate—5 mm/l.</i>						
268	6.9	0.91	21.2	9.42	3.69	6.64	6.09		
269	6.7	0.53	21.8	10.30	2.16	8.67	6.91		
271	8.2	0.68	20.2	8.84	1.95	7.57	6.60	.018	.027
275	6.5	0.72	18.2	7.93	2.15	6.50	5.45	.016	.026
Av.	7.1	0.71	20.4	9.12	2.49	7.35	6.26	.017	.027

\* Designates labelled carbon

The results of unlabelled pyruvate utilization measurements in ventricle slices from 9 normal rats are presented in table 6. The addition of pyruvate produced a 73 per cent increase in oxygen consumption. If the total amount of pyruvate which disappears were oxidized to  $CO_2$  and  $H_2O$ , the  $Q_{O_2}$  would have been 18.1 ( $2.5 \times -Q$  pyruvate) instead of 15.4. This does not allow however for the pyruvate which was converted to lactate. The oxygen equivalent for complete oxidation of the pyruvate disappearing, corrected for lactate appearance, is 12.6 or 81 per cent of the total observed oxygen consumption.

Similar data on 4 normal animals using pyruvate labelled with  $C^{14}$  in the alpha carbon position are shown in table 7. The amount of pyruvate which is oxidized to

CO<sub>2</sub> is calculated from the percentage of total counts in the incubating medium that appeared in the respiratory CO<sub>2</sub>. This calculation involves the assumption that for every radioactive carbon atom which appears as CO<sub>2</sub>, two non-radioactive carbon atoms from pyruvate are also converted to CO<sub>2</sub>. The amount of pyruvate oxidized to CO<sub>2</sub> accounts for 85 per cent of the net pyruvate disappearance and for 77 per cent of the total oxygen consumption of the tissue. The amount of pyruvate which is incorporated into the total fat and protein of the tissue represents less than one per cent of the net pyruvate disappearance. These results indicate that, of the total amount of pyruvate which disappears from ventricle slices, approximately 20 per

TABLE 8. UTILIZATION OF C<sup>14</sup> LABELLED ACETATE IN RAT VENTRICLE SLICES

RAT NO.	-Q <sub>O<sub>2</sub></sub>	-Q <sub>O<sub>2</sub></sub>	*CO <sub>2</sub> -Q <sub>ACETATE</sub>	*FAT -Q <sub>ACETATE</sub>	*PROTEIN -Q <sub>ACETATE</sub>
	No Substrate	Acetate—5 mM/l.			
268	6.9	12.9	2.84		
269	6.7	8.6	2.30		
271	8.2	12.2	3.06		
272	4.8	14.8	5.80	.005	.002
275	6.5	12.4	4.94	.004	.009
Av.	6.6	12.2	3.79	.005	.006

\* Designates labelled carbon

TABLE 9. COMPETITION FOR OXIDATION BETWEEN PYRUVATE AND ACETATE IN HEART MUSCLE

RAT NO.	-Q <sub>O<sub>2</sub></sub>	-Q <sub>O<sub>2</sub></sub>	-Q <sub>O<sub>2</sub></sub>	*CO <sub>2</sub> -Q <sub>ACETATE</sub>	-Q <sub>O<sub>2</sub></sub>	*CO <sub>2</sub> -Q <sub>ACETATE</sub>
	No Substrate	Pyruvate 5 mM/l.	Acetate—8.3 mM/l.		Acetate—8.3 mM/l. Pyruvate—5.0 mM/l.	
242	9.4	21.1	15.7	3.62	16.8	1.41
243	7.1	15.6	13.5	2.90	18.1	0.99
244	6.5	16.4	11.8	2.18	15.9	0.98
249			10.7	2.17	19.0	1.12
Av.	7.7	17.7	12.9	2.72	17.5	1.13

\* Designates labelled carbon

cent is converted to lactate, 69 per cent is oxidized to CO<sub>2</sub>, 1 per cent is incorporated into fat and protein and only 10 per cent is unaccounted for. When pyruvate is supplied to surviving rat ventricle slices it would appear that the major part of the respiratory process is involved in the utilization of pyruvate.

#### *Utilization of C<sup>14</sup> Labelled Acetate*

The rate of acetate oxidation to CO<sub>2</sub> was studied using acetate labelled with C<sup>14</sup> in the carboxyl position. Table 8 presents the data from experiments with 5 normal animals. It is apparent that acetate is readily oxidized by rat heart slices. The oxygen equivalent of the acetate oxidation ( $2 \times -Q$  acetate) in these experiments represents approximately 62 per cent of the total respiration. Additional data

of a similar character included in table 9 yielded the value of 40 per cent for the oxygen equivalent of acetate oxidation. The incorporation of radioactive carbon from acetate into the tissue fat and protein was so small that the results are of questionable significance.

Since cardiac muscle utilizes both acetate and pyruvate, the metabolism of the tissue in the presence of both substrates was studied. The question presented was whether the effect of each substrate would be additive or substitutive. The oxygen consumption and radioactive  $\text{CO}_2$  production were measured in experiments with carboxyl labelled acetate and unlabelled pyruvate added singly and together. The results of 4 such experiments are presented in table 9.

It was found that in the presence of pyruvate the oxidation of acetate to  $\text{CO}_2$  amounted to only about 40 per cent of that observed in the absence of pyruvate. From this it may be inferred that in heart muscle pyruvate and acetate participate to some extent in the same metabolic pathway.

Further experiments would be required to establish the quantitative relations of the competition suggested by these preliminary observations.

#### SUMMARY

Factors affecting the measurement of respiration of rat ventricle slices are discussed. Measurements of the utilization of labelled pyruvate by rat ventricle slices indicate that, of the total amount of pyruvate which disappears, approximately 69 per cent is oxidized to  $\text{CO}_2$ , 20 per cent is reduced to lactate and less than 1 per cent is incorporated into tissue fat and protein.

Labelled acetate added alone is readily oxidized to  $\text{CO}_2$  by rat ventricle and accounts for 40 to 60 per cent of the oxygen consumption. When pyruvate and acetate are added together both are metabolized but the oxygen consumption is the same as with pyruvate alone. The conversion of acetate to  $\text{CO}_2$  in the presence of pyruvate is less than half that observed when acetate was the only added substrate.

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# METABOLISM OF CARDIAC MUSCLE: UTILIZATION OF C<sup>14</sup> LABELLED PYRUVATE AND ACETATE IN DIABETIC RAT HEART AND DIAPHRAGM<sup>1</sup>

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ON THE basis of *in vitro* respiratory studies of dog skeletal and cardiac muscle, Shorr (1) concluded that the defect in carbohydrate metabolism resulting from insulin lack primarily involves the oxidative rather than the glycolytic reactions. It appeared from Shorr's experiments that cardiac tissue slices from diabetic dogs utilized pyruvate and lactate less readily than those from normal animals. Krebs (2) demonstrated an *in vitro* effect of insulin on the respiration of pigeon breast muscle brei, and Rice and Evans (3) found that this effect of insulin was associated with an increased utilization of pyruvate. Attempts to demonstrate a similar effect of insulin in mammalian muscle have failed (4). If the effect of insulin on the hexokinase reaction (5) is the major site of action of this hormone, it might be anticipated that pyruvate would be utilized in the normal manner by diabetic tissues.

It was therefore of interest to compare the utilization of pyruvate in diabetic and normal tissues and to supplement the respiratory findings with chemical balance studies of pyruvate. Sodium pyruvate labelled with C<sup>14</sup> in the alpha position was prepared so that the extent of the oxidation of pyruvate to CO<sub>2</sub> and of its participation in fat and protein metabolism could be followed. Similar experiments with diaphragm in place of cardiac tissue were also carried out. The utilization of sodium acetate labelled with C<sup>14</sup> in the carboxyl position was also measured for comparative purposes.

## METHODS

The methods for measuring respiration and substrate utilization have been described in the preceding paper (6). The animals used for the study of diaphragm muscle were approximately 200 gm. in weight so that the muscle was approximately the same thickness (ca. 0.4 to 0.5 mm.). The diaphragm was carefully dissected free of fat and connective tissue and one quarter of a diaphragm was used in each flask. The animals were fed Purina Dog Chow *ad libitum* and were not fasted before death.

Diabetic animals were prepared by the injection of alloxan administered subcutaneously (200 mg/kg. of body weight) or intravenously (40 mg/kg.). Only animals with continued, severe glycosuria and hyperglycemia were used. These animals exhibited pronounced polyphagia, polydipsia and polyuria, but did not develop gross ketonuria. In general, these animals maintained their body weight and appeared healthy.

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## RESULTS

*Metabolism of Unlabelled Pyruvate.* Preliminary experiments were performed with unlabelled pyruvate. Table 1 presents the results of respiration and pyruvate utilization measurements in cardiac slices from 11 diabetic animals. The average rate of respiration of diabetic heart slices in the absence of added substrate is approximately 28 per cent higher than with normal slices. The addition of 5 mm/l. of pyruvate

TABLE 1. UTILIZATION OF PYRUVATE BY DIABETIC RAT VENTRICLE

RAT NO.	DURATION OF DIABETES	BLOOD SUGAR	$-Q_{O_2}$	$Q_{LACTATE}^1$	$-Q_{O_2}$	$-Q_{PYRUVATE}^2$	$Q_{LACTATE}$	NET $-Q_{PYRUVATE}$ 0 INSULIN <sup>3</sup>	NET $-Q_{PYRUVATE}$ + INSULIN <sup>3</sup>
	Days	mg.-%	No substrate		Pyruvate 5 mm/l.				
10	10		11.5	1.43	15.6	5.15	4.25	2.33	2.03
11	16	450	8.7	1.36	14.1	5.08	4.59	1.85	
12	14	250	8.4	0.60	14.7	4.56	2.79	2.37	2.92
13	10	375	10.9	1.20	13.4	5.62	2.94	3.88	4.07
14	24	600	11.0	1.56	15.7	7.00	4.57	3.99	3.87
15	31	400	11.5	1.21	15.2	5.83	4.59	2.45	2.77
16	67	400	10.4	1.61	13.7	5.97	4.97	2.61	
17	57	530	13.5	1.63	13.4	5.51	5.60	1.54	
18	52	500	12.5	1.13	13.8	6.19	4.51	2.81	
19	55	525	13.7	1.51	15.6	7.75	5.96	3.30	
20	57	525	13.2	1.69	15.3	6.72	5.37	3.04	
Av.		455	11.4	1.36	14.6	5.94	4.56	2.74	3.13
S.E.M.			$\pm 0.5$	$\pm 0.10$	$\pm 0.3$	$\pm 0.28$	$\pm 0.30$	$\pm 0.23$	$\pm 0.38$
Av. 9 normal rats			8.9	0.69	15.4	7.22	2.86	5.05	
S.E.M.			$\pm 0.6$	$\pm 0.07$	$\pm 1.0$	$\pm 0.52$	$\pm 0.17$	$\pm 0.43$	
t <sup>4</sup>			3.21	5.50	0.77	2.17	4.92	5.78	

<sup>1</sup>  $Q_{Lactate}$  = microliters of lactate produced/mg. of dry tissue/hr.

<sup>2</sup>  $-Q_{Pyruvate}$  = microliters of pyruvate disappearing/mg. of dry tissue/hr.

<sup>3</sup> 'Iletin,' 200 gamma/flask was utilized except in *Exper. 15* in which 100 gamma per flask of crystalline insulin 'Lilly' was added.

<sup>4</sup>  $t$  = the significance factor of Fisher (FISHER, R. A. *Statistical Methods for Research Workers*. (4th ed.) Edinburgh: 1932).

produces a 28 per cent increase in the rate of oxygen consumption of diabetic heart in contrast to the 73 per cent increase in normal heart muscle, although the  $-Q_{O_2}$  in the presence of pyruvate is approximately the same for diabetic and normal slices.

The total pyruvate disappearance ( $-Q_{pyruvate}$ ) is 18 per cent lower in the diabetic than in the normal animals. The pyruvate disappearance corrected for lactate production (Net  $-Q_{pyruvate}$ ) (6) is 46 per cent lower in the diabetic animals. The addition of insulin *in vitro* in 5 experiments produced no significant effect on the pyruvate utilization of diabetic ventricle slices.

These results indicate that the *in vitro* utilization of pyruvate is depressed in diabetic cardiac muscle. In order to determine whether this disturbance of metabolism was peculiar to cardiac muscle, similar experiments were performed using rat diaphragm muscle. The results are presented in table 2. It is apparent that the *in vitro* utilization of pyruvate is depressed in diabetic rat diaphragm.

*Metabolism of Labelled Pyruvate.* Since the chemical measurement of pyruvate disappearance does not indicate the end products of its metabolism, it was of interest to determine directly the amount of pyruvate oxidized to  $\text{CO}_2$ . The above experiments were repeated using pyruvate labelled with  $\text{C}^{14}$  in the alpha position and measuring

TABLE 2. PYRUVATE UTILIZATION IN NORMAL AND DIABETIC RAT DIAPHRAGM

EXPER. NO.	DURATION OF DIABETES	BLOOD SUGAR	$-Q_{O_2}$	$Q_{LACTATE}$	$-Q_{O_2}$	$-Q_{PYRU-VATE}$	$Q_{LACTATE}$	NET $-Q_{PYRU-VATE}$
	Days	mg.%	No substrate		Pyruvate 5mM/l.			
Diabetic								
204	10	375	6.3	0.36	7.7	2.92	1.70	1.58
205	4	350	6.4	0.37	7.0	3.00	1.67	1.70
208	30	400	6.3	0.76	7.0	2.30	1.87	1.19
216	67	400	6.7	0.69	7.5	2.57	2.14	1.12
Av.			6.4	0.55	7.3	2.70	1.85	1.40±.14
Normal								
209			6.0	0.15	7.7	3.58	1.33	2.48
210			7.8	0.09	8.3	2.84	1.57	1.36
211			8.3	0.06	9.3	4.04	1.80	2.30
212			7.2	0.37	10.0	3.51	1.99	1.89
215			5.8	0.43	5.9	5.38	2.59	3.22
Av.			7.0	0.22	8.2	3.87	1.86	2.25±.31

the total activity in the respiratory  $\text{CO}_2$ . The oxidation of pyruvate (designated as  $-\text{Q}_{\text{pyruvate}}^{\text{CO}_2}$ ) was calculated from the percentage of the total radioactivity in the flask as pyruvate that appeared in the respiratory  $\text{CO}_2$ , assuming that for each  $\text{C}^{14}$  atom, two additional unlabelled C atoms from pyruvate appeared in the respiratory  $\text{CO}_2$ . The data are presented in tables 3 and 4. The radioactivity incorporated into the total fat ( $-\text{Q}_{\text{pyruvate}}^{\text{fat}}$ ) and protein ( $-\text{Q}_{\text{pyruvate}}^{\text{protein}}$ ) fractions of the tissue was also measured, and for convenience in making comparisons with analytical data, is expressed as microliters of pyruvate per milligram of dry tissue per hour.

The oxidation of pyruvate to  $\text{CO}_2$  is markedly depressed in the diabetic heart and diaphragm. It accounts for 80 to 85 per cent of the non-lactate pyruvate disappearance in both normal and diabetic heart slices, whereas it accounts for only 55 and 39 per cent in normal and diabetic diaphragm respectively. The incorporation of  $\text{C}^{14}$  from pyruvate into fat and protein is approximately the same for diabetic and normal tissues, although the percentage of the total pyruvate disappearing which is thus converted is higher in the diabetic than normal tissues. Approximately five times

as much  $C^{14}$  from pyruvate is incorporated into fat plus protein of diaphragm muscle as compared to heart slices. In other experiments (7) carried out by Dr. Villee of this laboratory it has been found that  $C^{14}$  from pyruvate is incorporated into glycogen

TABLE 3. UTILIZATION OF  $C^{14}$  LABELLED PYRUVATE IN DIABETIC RAT VENTRICLE

RAT NO.	$-Q_{O_2}$	$Q_{LACTATE}$	$-Q_{O_2}$	$-Q_{PYRUVATE}$	$Q_{LACTATE}$	$-Q_{PYRUVATE}$	$^{14}CO_2$ $-Q_{PYRUVATE}$	$^{14}FAT$ $-Q_{PYRUVATE}$	$^{14}PROTEIN$ $-Q_{PYRUVATE}$
	No substrate		Pyruvate 5 mm/l.						
Diabetic									
265	12.8	1.12	19.9	8.86	4.07	5.92	4.42		
266	9.1	1.12	13.2	6.20	3.81	3.52	2.39		
267	12.5	1.38	16.6	6.80	3.36	4.82	3.68	.019	.041
270	11.0	1.07	17.7	6.61	2.82	4.86	4.05	.029	.057
273	12.1	1.05	17.6	7.74	3.45	5.34	4.49	.029	.061
274	10.6	0.64	17.9	7.06	2.64	5.06	4.81	.019	.068
Av.	11.4	1.06	17.2	7.21	3.36	4.92	3.87	.024	.057
S.E.							$\pm 0.12$		
Diabetic + Insulin <sup>1</sup>									
267	13.0	1.05	17.6	7.57	3.26	5.36	3.36	.019	.059
270	11.5	0.97	18.2	7.14	3.02	4.91	4.54	.111	.111
273	13.4	0.99	15.0	7.14	3.64	4.49	4.00	.030	.065
274	9.6	0.60	18.5	7.65	2.91	5.34	4.84	.016	.071
Av.	11.9	0.86	17.3	7.38	3.21	5.03	4.19	.044	.078
S.E.							$\pm 0.32$		
4 Normal									
Av.	7.1	0.71	20.4	9.12	2.49	7.35	6.26	.017	.027
S.E.							$\pm 0.32$		
4 Normal + Insulin <sup>1</sup>									
Av.	7.7	0.62	19.5	8.65	2.36	6.90	6.46	.020	.036
S.E.							$\pm 0.38$		

The duration of diabetes in these animals varied from 5 to 28 days with an average of 14 days. The blood sugar averaged 385 mg. %.

\* Designates labelled carbon.

<sup>1</sup> 200 gamma of crystalline insulin ('Lilly') were added per flask.

in normal diaphragm muscle in amounts corresponding to approximately 5 to 10 per cent of the total pyruvate disappearing.

The addition of insulin *in vitro* had no significant effect on the utilization of pyruvate in either normal or diabetic rat heart slices. In diabetic diaphragm, insulin *in vitro*, increased the oxidation of pyruvate 37 per cent, but was without significant effect in normal diaphragm. No significant effect of insulin on the incorporation of pyruvate into fat and protein of the tissues was observed in the present experiments.

*Metabolism of Labelled Acetate.* Since the oxidation of fat is believed to proceed in normal fashion in diabetic animals, it was of interest to compare the utilization of a

TABLE 4. UTILIZATION OF C<sup>14</sup> LABELLED PYRUVATE IN RAT DIAPHRAGM

RAT NO.	-Q <sub>O<sub>2</sub></sub>	-Q <sub>PYRUVATE</sub>	Q <sub>LACTATE</sub>	NET -Q <sub>PYRUVATE</sub>	*CO <sub>2</sub> -Q <sub>PYRUVATE</sub>	*FAT -Q <sub>PYRUVATE</sub>	*PROTEIN -Q <sub>PYRUVATE</sub>
Pyruvate 5 mM/l.							
<i>Diabetic</i>							
273	5.5	3.82	2.33	1.99	0.54	0.127	0.236
274	6.0	3.01	1.54	1.75	0.53	0.146	0.310
278	5.6	2.08	1.63	0.92	0.78	0.068	0.340
280	6.3	2.32	1.73	1.06	0.56	0.036	0.240
Av.	5.9	2.81	1.81	1.43	0.60	0.094	0.282
S.E.					±0.06		
<i>Diabetic + Insulin</i>							
273	6.6	3.34	1.24	2.60	0.78	0.087	0.359
274	6.7	2.75	1.01	1.96	0.78	0.144	0.330
278	5.7	3.02	1.59	2.14	1.00	0.031	0.195
280	6.3	2.46			0.72	0.049	0.216
Av.	6.3	2.89	1.25	2.23	0.82	0.078	0.275
S.E.					±0.06		
<i>Normal</i>							
275	6.4	4.44	1.67	3.03	2.32	0.123	0.179
276	7.2	5.33	0.73	4.76	2.19	0.059	0.294
279	6.2	2.79	1.34	1.90	1.30	0.045	0.258
282	7.3	5.05	1.39	3.90	1.65	0.079	0.145
Av.	6.8	4.40	1.28	3.40	1.87	0.077	0.219
S.E.					±0.22		
<i>Normal + Insulin</i>							
275	7.5	4.44	1.74	3.03	2.29	0.115	0.300
276	8.2	4.86	0.58	4.40	2.29	0.036	0.370
279	7.4	3.13	1.36	2.21	1.68	0.048	0.262
282	6.9	4.19	1.39	3.27	1.42		0.140
Av.	7.5	4.16	1.27	3.23	1.92	0.066	0.268
S.E.					±0.22		

\* Designates labelled carbon.

The average duration of diabetes in these animals was 12 days. The average blood sugar at death was 389 mg. %.

2 carbon substrate, such as acetate, under conditions similar to those for pyruvate. Acetate labelled with C<sup>14</sup> in the carboxyl position was used for this purpose. Oxidation of acetate to CO<sub>2</sub> was determined by measuring the radioactivity in the respiratory CO<sub>2</sub>. The radioactivity in the total fat and protein fractions of the tissue was also measured. The data are presented in tables 5 and 6. It appears from the data that acetate is

oxidized as readily in diabetic as in normal heart slices. In diabetic diaphragm, however, acetate oxidation is depressed 43 per cent below that of normal diaphragm. Addition of insulin *in vitro* had no effect on acetate oxidation in either tissue. The incorporation of acetate into fat and protein is very small for both heart and dia-

TABLE 5. UTILIZATION OF  $C^{14}$  LABELLED ACETATE IN DIABETIC RAT VENTRICLE

RAT NO.	$-Q_{O_2}$	$-Q_{O_2}$	$^{*}CO_2$ $-Q_{ACETATE}$	$^{*}FAT$ $-Q_{ACETATE}$	$^{*}PROTEIN$ $-Q_{ACETATE}$
	No substrate	Acetate 5 mm/l.			
Diabetic					
265	12.8	17.2	5.05		
266	9.1	14.8	2.31		
270	11.0	13.4	2.52		
273	21.1	13.1	5.16	.004	.006
274	10.6	16.2	6.50	.003	.004
Av.	11.1	14.9	4.31	.004	.005
S.E.			$\pm 0.82$		
Diabetic + Insulin					
267	12.5	13.9	2.36		
270	11.5	13.2	2.74		
273	13.4	15.0	5.45	.002	.007
274	9.6	15.7	6.20	.002	.005
Av.	11.8	14.5	4.19	.002	.006
S.E.			$\pm 0.74$		
5 Normal Rats					
Av.	6.6	12.2	3.79	.005	.006
S.E.			$\pm 0.67$		
5 Normal Rats + Insulin					
Av.	7.5	11.8	3.47	.003	.006
S.E.			$\pm 0.52$		

\* Designates labelled carbon.

The average duration of diabetes in these animals was 15 days.

The average blood sugar at death was 376 mg. %.

phragm. The values given in the table are of questionable significance since the counts were only slightly above background level.

#### DISCUSSION

The experiments presented above were planned to add information on the rôle that insulin plays in the metabolism of pyruvate, a key intermediary metabolite. The observation that the heart and diaphragm of diabetic animals have a diminished ability to metabolize added pyruvate is in agreement with the results of Shorr who studied the lactate and pyruvate metabolism of depancreatized dog heart slices.

It would further appear from our observations that the utilization of the pyruvate which is metabolized by the heart and diaphragm of diabetic animals is abnormal

TABLE 6. UTILIZATION OF C<sup>14</sup> LABELLED ACETATE BY RAT DIAPHRAGM MUSCLE

RAT NO.	-Q <sub>O<sub>2</sub></sub>	*CO <sub>2</sub> -Q <sub>ACETATE</sub>	*FAT -Q <sub>ACETATE</sub>	*PROTEIN -Q <sub>ACETATE</sub>
<i>Acetate 5 mm/l.</i>				
<i>Diabetic</i>				
273	6.1	1.67	.001	.008
274	6.6	2.28	.003	.023
278	4.9	1.61	.000	.020
280	6.4	2.26	.002	.024
281	5.9	1.56	.005	.015
Av.	6.0	1.88	.002	.018
S.E.		±0.16		
<i>Diabetic + Insulin</i>				
273	6.4	1.60	.003	.018
274	6.0	1.66	.003	.030
278	5.6	1.62	.000	.011
280	6.1	2.29	.003	.020
281	5.6	1.53	.004	.022
Av.	5.9	1.74	.003	.020
S.E.		±0.14		
<i>4 Normal</i>				
275	6.6	3.85	.011	.044
276	6.5	3.36	.001	.028
279	6.0	2.66	.000	.016
282	6.8	3.32	.000	.016
Av.	6.5	3.30	.003	.026
S.E.		±0.24		
<i>4 Normal + Insulin</i>				
275	6.1	3.17	.009	.016
276	7.2	3.54	.003	.028
279	7.4	3.07	.000	.017
282	7.0	3.34	.004	.030
Av.	6.9	3.28	.004	.023
S.E.		±0.10		

\* Designates labelled carbon.

The average duration of diabetes in these animals was 11 days.

The average blood sugar at death was 402 mg. %.

in two other respects: 1) a greater proportion of the pyruvate is converted to lactate than in normal tissue and 2) a smaller proportion of the pyruvate is converted to CO<sub>2</sub>.

Oxidation of acetate to CO<sub>2</sub> is unchanged from normal in diabetic heart muscle, but diminished in diabetic diaphragm. There is no obvious explanation for this differ-

ence. It would be of interest to study the oxidation of a 4-carbon metabolite, such as acetoacetate in normal and diabetic muscle.

Added insulin had no significant effect in correcting these metabolic abnormalities save in one series of experiments, namely, the diaphragms of diabetic animals. In this series, insulin increased the  $\text{CO}_2$  production coming from the labelled pyruvate, increased the net pyruvate utilized, and diminished the lactate production. The addition of insulin did not, however, restore these quantities to their normal values, nor were any insulin effects demonstrable in cardiac muscle. Whether this is due to failure to obtain an adequate intracellular concentration of insulin or some other factor is not known.

At all events, it would appear that in insulin insufficiency, produced by alloxan, there is diminished ability of cardiac and diaphragm muscle to carry out the steps required to convert pyruvate to  $\text{CO}_2$ .

#### SUMMARY

The metabolism of pyruvate and acetate in cardiac and diaphragm muscle of normal and diabetic rats has been studied, *in vitro*. Oxygen consumption, pyruvate utilization, lactate production and the  $\text{C}^{14}\text{O}_2$  production from  $\text{CH}_3\text{C}^{14}\text{OCOOH}$  and  $\text{CH}_3\text{C}^{14}\text{OOH}$  have been measured.

Diminished pyruvate utilization has been observed in both cardiac and diaphragm muscle of diabetic animals. Addition of insulin, *in vitro*, increased the conversion of labelled pyruvate to  $\text{C}^{14}\text{O}_2$  in the diaphragms of the diabetic animals but not in cardiac muscle. Diminished acetate utilization has been observed in diabetic diaphragm muscle, but was unchanged from normal in diabetic heart muscle. Addition of insulin, *in vitro*, had no effect on the conversion of labelled acetate to  $\text{C}^{14}\text{O}_2$ . Measurement of the radioactivity of fat and protein fractions revealed that  $\text{C}^{14}$  incorporation from  $\text{CH}_3\text{C}^{14}\text{OCOOH}$  was five-fold greater in diaphragm than in cardiac muscle.

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# METABOLISM OF THE HEART IN RELATION TO DRUG ACTION<sup>1</sup>. IV. EFFECTS OF VARIOUS SUBSTRATES UPON THE ISOLATED PERFUSED RAT HEART

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THE mammalian heart has been shown to be capable of utilizing various substrates. This conclusion is based upon measurements of increased oxygen utilization and substrate disappearance in various types of surviving beating heart preparations (heart-lung, heart-oxygenator, isolated perfused, etc.), and in heart tissue preparations (slices, minces, homogenates, etc.) (see references in discussion). There are very few data, however, on the effects of many of these substrates on the functional activity of the 'normal' isolated perfused heart, or on the activity of the isolated perfused heart which has been made hypodynamic by perfusion with a substrate-free medium. As part of an investigation of the metabolic actions of various cardiac drugs, we have studied *a*) the effects of various concentrations of a number of possible substrates on the 'normal' isolated perfused rat heart, and *b*) the relative abilities of these substances to restore the activity (measured chiefly by amplitude of contraction) of the heart following prolonged perfusion on a substrate-free medium.

## METHODS

Rat hearts were perfused by the Langendorff method through the aorta, at a perfusion pressure of 24 cm. of water. Male albino rats (200-300 gm.) were decapitated, the heart cannulated<sup>3</sup>, rapidly excised, and attached to the perfusion apparatus. It was essential that the perfusion be started within 3 minutes, in order to avoid early failure. The heart was suspended in the central air-well of an ordinary glass angel-food cake baking dish in the central part of which a hole had been drilled. The dish served as a constant temperature bath which was maintained at 37.5°C., and the perfusion fluid was warmed to this temperature by circulation through a glass coil immersed in the bath. The perfusion was carried out by attaching one end of this coil to the cannula through the center hole of the dish (fig. 1). The perfusion medium used was that described by Chenoweth and Koelle (1) and was aerated with 95 per cent oxygen—5 per cent carbon dioxide. The standard medium used contained 0.01 M glucose. The various solutions to be tested were connected by means of a five-way attachment to the warming coil, and when the solutions were changed the previous solution was first drained

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<sup>3</sup> Heparin was added to the medium in the cannula to decrease the likelihood of clot formation before the perfusion was begun.

from the coil to an outside receiver, in order that the new solution would reach the heart within a period of one minute.

The contractions of the heart were recorded with a Gimble-type lever, using either smoked paper or an ink-writer.<sup>4</sup> Approximately one hour was required for the activity of the heart to become constant, and no experiments were performed until constant activity had been observed for a period of at least 15 minutes. Heart rate and coronary flow were measured, but since the changes could not be correlated with other data, the values are not reported.

The substrates used were previously described (2) except for oxalacetic acid (Bios) and  $\beta$ -hydroxybutyric acid (Bios). All substrates were used as the sodium salts, neutralized to pH 7.5. All media were prepared fresh each day with redistilled water from a concentrated stock solution containing no glucose or sodium bicarbonate.

Each substrate was tested on at least two hearts in both types of experiments described below and on at least three hearts if a pronounced effect was observed.

The following two general types of experiments were carried out.

**PART I. *Direct Effects of Substrates.*** Following the initial equilibration period with the standard glucose medium, the latter was replaced by the test medium containing various concentrations of substrate in place of glucose. The test medium was ordi-

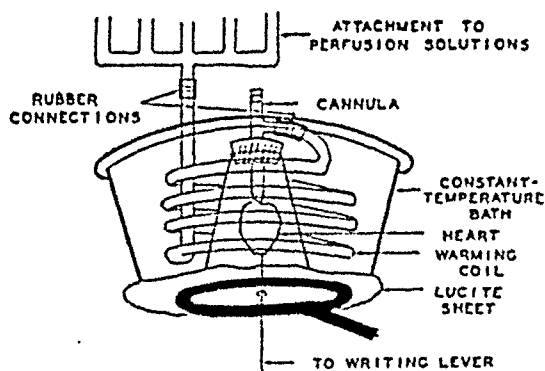


Fig. 1. SCHEMATIC DIAGRAM of perfusion apparatus

narly perfused for 5 minutes after which standard glucose medium was again perfused for 10 to 15 minutes, or for a longer period if necessary to attain a constant level of activity. The other concentrations of substrates were then tested in the same manner. Usually the lowest concentration was tested first, and the successively higher concentrations subsequently. Recovery on the standard glucose medium following depression with a substrate varied with the substrate and the concentration tested, but further substrate testing was frequently carried out in spite of failure to recover completely on glucose. There was, in general, a slow decline in amplitude of contraction even on the standard glucose medium, but this decline did not significantly affect the results obtained during the short test periods.

**PART II. *Relative Abilities of Substrates to Restore the Activity of the Substrate-Depleted Heart.*** Most of these experiments were carried out in the following manner: Following the initial equilibration period with the standard glucose medium, the latter was replaced by the same medium containing no glucose or other substrate, and per-

<sup>4</sup> In experiments through No. 74 recording was done on smoked paper, and in all subsequent experiments a considerably lighter ink writer was used. The generally greater amplitudes shown for the later experiments are due to the use of this lighter lever writing with less friction, and do not signify a more active heart preparation.

fusion on this substrate-free medium was continued until the amplitude of contraction had decreased to about one-third the initial value. The glucose-free medium was then replaced by a medium containing various concentrations of the substrate under investigation. Each concentration was usually tested for a 5-minute period, and then replaced by the standard glucose medium, in most cases for about 10 to 15 minutes, or until a constant level of activity was attained. The lowest concentrations were usually tested first, followed by successively higher concentrations. One hour or longer was usually required for the amplitude to decrease to one-third its initial value, when perfused with substrate-free medium for the first time, but subsequently the desired percentage depression occurred in 10 minutes or less, due probably to relative exhaustion of endogenous substrate during the first prolonged perfusion.

In addition to the above experiments which were carried out on all of the substrates, further investigations were made with those substrates which produced some recovery following the substrate-free perfusion, in order to determine the extent and duration of recovery following depression to nearly zero amplitude of contraction.

## RESULTS AND DISCUSSION

**PART I. *Direct Effects of Substrates.*** Typical results obtained when the standard glucose medium was replaced by various concentrations of different substrates usually for 5-minute periods are given in table 1. The amplitude of contraction on the standard glucose medium immediately before perfusion with the test substrate is given for each concentration tested, as the level of activity of the heart may be of importance with respect to the nature and magnitude of the response to a given substance. The substrate concentrations were tested in the order given in the table.

It is seen in table 1 that none of the substrates tested increased the amplitude of contraction of the heart at any of the concentrations tested. Glutamate at several concentrations between 0.0005 M and 0.01 M did increase the amplitude of contraction by as much as 50 per cent in several experiments, but the effect was not obtained consistently and its significance is uncertain at present. Most of the other substrates produced varying degrees of depression with increasing concentration, which was sometimes overcome by subsequent perfusion on the glucose medium, and sometimes not. An attempt was made to test the substances over a wide range of concentrations, in order to determine the concentrations producing no effect, those producing a threshold effect, and those producing a pronounced effect. Forssman and Lindsten (3) found that succinate and malonate frequently increased slightly the contraction amplitude of isolated perfused rabbit hearts, and that citrate,  $\alpha$ -ketoglutarate, aconitate, and glutamate produced either no effect or depression at the various concentrations tested.

**PART II. *Relative Abilities of Substrates to Restore the Activity of the Substrate-Depleted Heart.*** The substrates investigated in Part I were next tested for their ability to restore the activity of the heart following prolonged perfusion on a substrate-free medium, as described under METHODS. Typical results are given in tables 2 and 3. The concentrations were tested in the order given in the tables, and were usually those which had no effect on the 'normal' perfused heart (see Part I). Table 2 gives those compounds with which no recovery was observed at any concentration tested,

TABLE I. ACTION OF VARIOUS SUBSTRATES ON THE AMPLITUDE OF CONTRACTION OF THE PERFUSED RAT HEART

EXP. NO.	TEST SUBSTRATE		AMPLITUDE		% OF A OBTAINED DURING TEST SUBSTRATE <sup>1</sup>	% OF A OBTAINED DURING GLUCOSE (AFTER TEST SUBSTRATE) <sup>1</sup>
	Substrate	Conc.	Following equilibration <sup>1</sup>	During glucose A		
63	Acetate	M	mm.	mm.		
			25 (75)			
		$5 \times 10^{-4}$		25	80	80
		$2 \times 10^{-3}$		18	61	61 (3)
		$1 \times 10^{-2}$		13	61 (2)	93 (5)
87	$\beta$ -hydroxy-butyrate	$5 \times 10^{-2}$		9	44 (1)	100 (2)
			75 (49)			
		$1 \times 10^{-4}$		75	99	100
		$1 \times 10^{-3}$		76	101	97
		$1 \times 10^{-2}$		72	97	93
72	Caprylate		22 (74)			
		$4 \times 10^{-2}$		22	91	77 (1)
		$2 \times 10^{-3}$		14	74	74 (1)
		$1 \times 10^{-4}$		9	78	89 (3)
		$5 \times 10^{-4}$		7	79	79 (9)
		$2 \times 10^{-3}$		6	0 (1)	0
						0 (60)
58	Citrate		30 (43)			
		$1 \times 10^{-4}$		30	107	97
		$5 \times 10^{-4}$		29	93	90
		$2 \times 10^{-3}$		26	81	89 (2)
91	Fumarate		80 (72)			
		$1 \times 10^{-3}$		80	106	111
		$1 \times 10^{-4}$		80	100	105
		$1 \times 10^{-2}$		64	109	103
		$1 \times 10^{-2}$		61	100	97
106	Glutamate		68 (57)			
		$5 \times 10^{-4}$		68	96	103
		$2 \times 10^{-3}$		67	94	94
		$1 \times 10^{-2}$		64	88	89
		$5 \times 10^{-2}$		57	70	81
82	Lactate		60 (89)			
		$1 \times 10^{-4}$		60	100	100 (2)
		$3 \times 10^{-4}$		57	90	90
		$1 \times 10^{-3}$		48	83	83
		$5 \times 10^{-3}$		41	66	56
61	L-Malate		43 (37)			
		$1 \times 10^{-4}$		43	93 (2)	86
		$5 \times 10^{-4}$		32	75	69 (3)
		$2 \times 10^{-3}$		19	47	58 (5)
		$1 \times 10^{-2}$		10	70	90
92	Oxalacetate	$5 \times 10^{-2}$		8	0	75 (3)
			76 (51)			
		$1 \times 10^{-4}$		76	92	94
		$5 \times 10^{-4}$		71	92	92
		$2 \times 10^{-3}$		64	95	92
		$1 \times 10^{-2}$		61	90	102

TABLE 1—Continued

EXP. NO.	TEST SUBSTRATE		AMPLITUDE		% OF A OBTAINED DURING TEST SUBSTRATE <sup>2</sup>	% OF A OBTAINED DURING GLUCOSE (AFTER TEST SUBSTRATE) <sup>2</sup>
	Substrate	Conc.	Following equilibration <sup>1</sup>	During glucose A		
54	Pyruvate	<i>M</i>	<i>mm.</i>	<i>mm.</i>		
			34 (53)			
		$1 \times 10^{-3}$		26	96	100
59	Succinate	$5 \times 10^{-3}$		26	61	100
		$1 \times 10^{-2}$		26	27 ( $1\frac{1}{2}$ )	96 (5)
			30 (14)			
		$5 \times 10^{-4}$		30	100	110
		$2 \times 10^{-3}$		36	97	100
		$1 \times 10^{-2}$		35	94	94 (7)
		$5 \times 10^{-2}$		31	26 (1)	100 (2)

<sup>1</sup>Amplitude after initial equilibration period, prior to first perfusion with a test substrate. The value in parentheses is the time in minutes of this initial period.

<sup>2</sup>All values are for the amplitude measured 4 minutes after the beginning of perfusion on the particular medium, unless otherwise indicated by the time in minutes given in the parentheses.

and table 3 those with which recovery was obtained at limited concentrations. It is seen in table 3 that glucose, pyruvate, acetate and  $\beta$ -hydroxybutyrate produced varying degrees of recovery at several concentrations following depression of the heart to one-fourth to one-half the initial amplitude while on substrate-free medium. It should be noted that the recovery produced by the latter three substrates was approximately one-half to two-thirds that subsequently obtained with the standard glucose medium.

Table 3 also gives the results of experiments with glucose, pyruvate, acetate, and  $\beta$ -hydroxybutyrate in which the perfusion on substrate-free medium was continued until the amplitude of contraction was almost zero before changing to the substrate medium, and it is seen that varying degrees of recovery were obtained under these circumstances.

Experiments were also carried out with the optimal concentrations of glucose, pyruvate, acetate and  $\beta$ -hydroxybutyrate following depression to almost zero amplitude of contraction by prolonged perfusion on substrate-free medium, in which the subsequent perfusion with the test solution was continued for longer periods to determine the relative duration of the recovery (fig. 2). It is seen that glucose produced the greatest recovery, and that the duration of the effect was much greater with glucose and pyruvate than with acetate or  $\beta$ -hydroxybutyrate.

Utilization of pyruvate by the dog heart has been shown by measurements of pyruvate disappearance in heart-lung, heart-oxygenator, and open chest preparations (4, 5), but evidence as to the ability of pyruvate to restore the activity of the substrate-depleted heart was inconclusive (4). Monkey hearts have been perfused with pyruvate and also with acetate as sole carbon sources, but no data were given as to their relative ability to maintain the heart compared to glucose (1). Our work indicates that pyruvate, although inferior to glucose as a substrate, will restore the activity of hearts that have been depressed to zero amplitude of contraction by substrate-free perfusion, and will maintain activity for a period of at least 3 hours.

Barcroft *et al.* (6, 7) were able to maintain the isolated perfused rabbit heart for a two to three times longer period with Locke's solution containing acetate as the sole carbon source than with a substrate-free Locke's solution, and showed that acetate disappeared from the perfusion medium. The isolated cat heart has been

TABLE 2. ACTION OF VARIOUS SUBSTRATES WHICH FAIL TO INCREASE THE AMPLITUDE OF CONTRACTION OF THE SUBSTRATE-DEPLETED RAT HEART

EXP. NO.	TEST SUBSTRATE		AMPLITUDE		% OF A OBTAINED DURING SUBSTRATE-FREE MEDIUM <sup>1</sup>	% OF A OBTAINED DURING TEST SUBSTRATE <sup>2</sup>	% OF A OBTAINED DURING GLUCOSE (AFTER TEST SUBSTRATE) <sup>3</sup>
	Substrate	Conc.	Following equilibration <sup>1</sup>	During glucose A			
		<i>M</i>	<i>mm.</i>	<i>mm.</i>			
74	Caprylate		35 (42)				
		$4 \times 10^{-2}$		16	56	31 (3)	56 (9)
		$2 \times 10^{-2}$		9	67	44 (1)	44
58	Citrate	$5 \times 10^{-1}$	30 (43)	9	78	0 (1)	55 (2)
55	Fumarate	$1 \times 10^{-2}$	33 (40)	19	37	21 (1)	53
57	Fumarate	$5 \times 10^{-1}$	38 (23)	38	21	5 (1)	65 (5)
69	Glutamate	$5 \times 10^{-1}$	28 (93)	20	45	30 (2)	75 (9)
		$2 \times 10^{-2}$		15	40	20 (2)	80 (9)
83	Lactate	$1 \times 10^{-1}$	85 (74)	85	24	11	21
		$3 \times 10^{-1}$		32	28	13	38 (10)
							38
							47 (9)
62	Malate	$1 \times 10^{-1}$	35 (52)	14	86	42 (2)	57
71	Oxalacetate	$1 \times 10^{-1}$	33 (86)	22	46	35 (1) <sup>3</sup>	
		$5 \times 10^{-1}$				41 (4) <sup>3</sup>	
		$2 \times 10^{-2}$				32 (2) <sup>3</sup>	68 (10)
		$5 \times 10^{-1}$		15	67	47	87 (5)
		$2 \times 10^{-2}$		13	54	39 (3)	
50	Succinate	$2 \times 10^{-2}$	34 (129)	26	58	44	100 (9)

<sup>1</sup> See footnote 1 to table 1.    <sup>2</sup> See footnote 2 to table 1.    <sup>3</sup> Solutions changed directly from one to another.

demonstrated to utilize acetate containing C<sup>13</sup> (8). Our experiments indicate that acetate can partially maintain the mechanical activity of the rat heart when it is the sole exogenous substrate present, but that it is much less effective than pyruvate or glucose in this respect.

Added  $\beta$ -hydroxybutyrate disappears from the blood in dog heart-lung preparations (9, 10), but these observations were not correlated with the maintenance of mechanical activity. We have demonstrated that  $\beta$ -hydroxybutyrate will stimulate

TABLE 3. ACTION OF VARIOUS SUBSTRATES WHICH INCREASE THE AMPLITUDE OF CONTRACTION OF THE SUBSTRATE-DEPLETED RAT HEART

EXP. NO.	TEST SUBSTRATE		AMPLITUDE		% OF A DURING SUB-STRATE-FREE MEDIUM <sup>2</sup>	% OF A OBTAINED DURING TEST SUBSTRATE <sup>3</sup>	% OF A OBTAINED DURING GLUCOSE (AFTER TEST SUBSTRATE) <sup>2</sup>
	Substrate	Conc.	Following equilibration <sup>1</sup>	DURING glucose A			
		<i>M</i>	<i>mm.</i>	<i>mm.</i>			
67	Acetate		34 (42)				
		$2 \times 10^{-5}$		20	35	30 (1) <sup>3</sup>	
		$1 \times 10^{-4}$				28 (1) <sup>3</sup>	
		$5 \times 10^{-4}$				55 <sup>3</sup>	80 (14)
76	Acetate	$5 \times 10^{-4}$		16	38	65	87 (14)
		$5 \times 10^{-4}$	65 (66)				
		$5 \times 10^{-4}$		45	24	69	91
		$2 \times 10^{-3}$		43	26	80 (7)	96 (9)
81	Acetate	$5 \times 10^{-4}$	65 (42)	85	26	55	
90	Acetate	$5 \times 10^{-4}$	81 (52)	81	1	6	10 <sup>4</sup>
100	Acetate	$5 \times 10^{-4}$	85 (72)	85	1	6 (8)	
88	$\beta$ -hydroxy-butyrate		76 (80)				
		$1 \times 10^{-4}$		76	24	22	37
		$1 \times 10^{-3}$		31	39	49	66
		$1 \times 10^{-2}$		22	45	73	86
101	$\beta$ -hydroxy-butyrate	$1 \times 10^{-2}$		15	53	80	80
			105 (53)				
		$1 \times 10^{-2}$		105	2	11 (2)	
102	$\beta$ -hydroxy-butyrate		52 (73)				
		$1 \times 10^{-2}$		52	4	14	
		$1 \times 10^{-4}$		22	36	23 (1) <sup>3</sup>	
		$5 \times 10^{-4}$				23 (2) <sup>3</sup>	
70	Pyruvate	$2 \times 10^{-3}$				55 <sup>3</sup>	73 (2)
			33 (73)				
		$5 \times 10^{-4}$		22	36	23 (1) <sup>3</sup>	
		$2 \times 10^{-3}$				55 <sup>3</sup>	
77	Pyruvate		67 (45)				
		$5 \times 10^{-4}$		49	41	31	63
		$2 \times 10^{-3}$		33	36	64	85 (3)
		$2 \times 10^{-3}$		28	29	46 (2)	68 (10)
78	Pyruvate	$2 \times 10^{-3}$	80 (61)	80	1	16	25
99	Pyruvate	$2 \times 10^{-3}$	105 (53)	105	1	10 (9)	
38	Glucose		28 (93)				
		$1 \times 10^{-2}$		28	32	72	
		$1 \times 10^{-2}$		20	35	92	
		$1 \times 10^{-2}$		18	25	94	
89	Glucose		61 (50)				
		$1 \times 10^{-2}$		61	2	30	

<sup>1</sup> See footnote 1 to table 1. <sup>2</sup> See footnote 2 to table 1. <sup>3</sup> Solutions changed directly from one to another. <sup>4</sup> Changed from acetate to pyruvate (10 min.) to glucose.

hearts partially depressed by perfusion on substrate-free medium, and will restore for a short period of time the activity of hearts which have been depressed to zero amplitude of contraction.

It is surprising that as little as 50 per cent depression of the heart perfused with substrate-free medium could not be overcome with lactate since previous work showed lactate was more effective than glucose in improving the functional activity of glycogen-depleted or failing dog hearts in the heart-lung preparation (11-13), and also

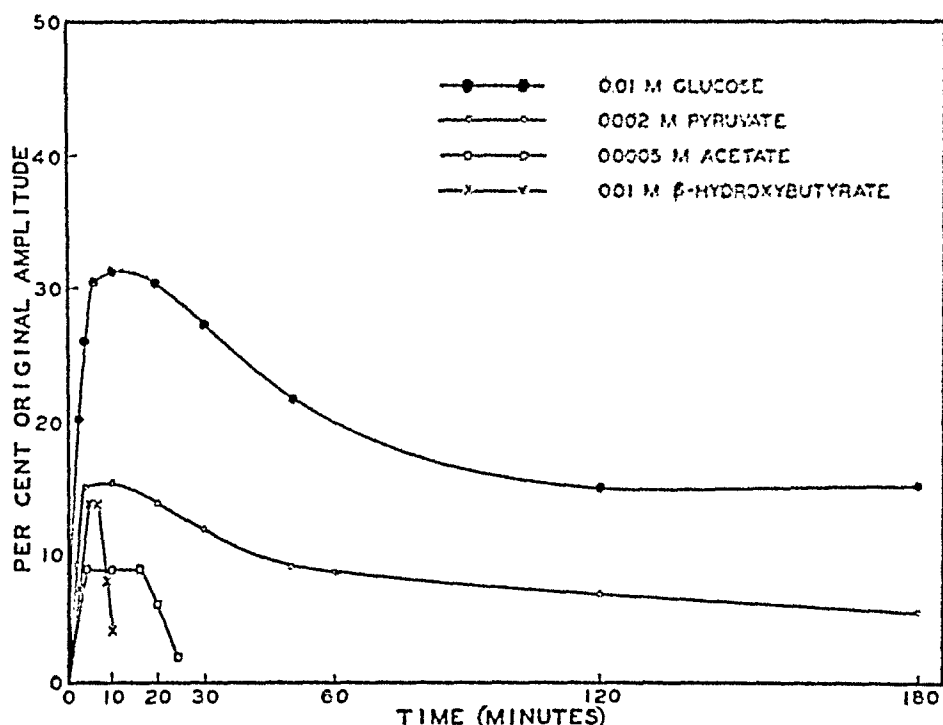


Fig. 2. RECOVERY of the substrate-depleted perfused rat heart with various substrates. Hearts were perfused on a substrate-free medium until the contraction amplitude had decreased to approximately 1% of its original value at the start of the experiment. Media containing the above substrates were substituted for the substrate-free medium at 0 time in the above figure.

because of the demonstrated utilization of lactate by rat heart slices (2) and homogenates (14). The product of lactate oxidation is in all probability pyruvate, which is capable of maintaining the activity of the rat heart for considerable periods of time (fig. 2). It occurred to us that failure of the heart to function on lactate might be due to a deficiency of coenzyme I, which is required for the oxidation of lactate. Break-down of coenzyme I in damaged tissues (15, 16) and anoxic heart muscle (17) and the inhibition of the enzymatic destruction of coenzyme I by nicotinamide (15, 16) have been described. Calder (18) reported that nicotinic acid (1:100,000 to 1:2,000,000) and nicotinamide (1:200,000) cause recovery of failing perfused rabbit hearts in the presence or absence of glucose; he suggested that failure may be due to destruction of coenzyme I as a result of anoxia incident to experimental technique, and that nicotinic acid or nicotinamide can overcome this deficiency. We investigated the effect of  $10^{-4}$  M (1:82,000) and  $10^{-5}$  M (1:820,000) nicotinamide on the ability of lactate

( $10^{-4}$  M,  $3 \times 10^{-4}$  M, and  $10^{-3}$  M) to restore the activity of hearts depressed by perfusion on substrate-free medium, and were unable to obtain a consistent effect, although in several instances slight recovery occurred. In these experiments the same concentration of nicotinamide was present in all perfusion solutions used with any one heart preparation. Additional evidence that coenzyme I deficiency may not be responsible for the failure of lactate to restore the activity of the rat heart may be inferred from the fact that a transient recovery was obtained with  $\beta$ -hydroxybutyrate, the oxidation of which to acetoacetate requires coenzyme I (19).

Our failure to obtain any recovery of activity of the partially substrate-depleted rat heart with the other compounds tested indicates that there is no correlation between the utilization of a substrate, as determined by measurements of oxygen-consumption of heart slices in the presence of the substrate (2), and the ability of the substrate to improve the functional activity of the heart. For example, succinate increased the  $Q_{O_2}$  of the respiring rat heart slices by as much as 350 per cent (2), and yet we were unable to demonstrate any effects of this substrate on the restoration of the substrate-depleted heart. Many other compounds were shown to increase the  $Q_{O_2}$  of heart slices without having any apparent effect on the functional activity of the perfused heart at various levels of activity.

The results reported in this paper may be due to effects either on the myocardium or on some nervous mechanism of the heart, as all of this work was done with spontaneously beating hearts. The restoration of the functional activity of the substrate-depleted heart by a substrate could result either from an increase in the activity of the pace-maker or other nervous structure, or from a direct effect on the myocardium itself, or perhaps both. We are now attempting to clarify these results by studying substrate effects on electrically-driven rat ventricle preparations and on isolated auricles.

#### SUMMARY

Pyruvate, acetate,  $\beta$ -hydroxybutyrate, lactate, succinate, fumarate, malate, oxalacetate, citrate, glutamate and caprylate have been investigated over a range of concentrations with respect to their action on the 'normal' isolated perfused heart. None of these substances increased the amplitude of contraction, and most of them depressed the heart at the higher concentrations tested. The relative abilities of the above substances and glucose to increase the contraction amplitude of the heart depressed by perfusion with a substrate-free medium were investigated.

Of the substrates tested, glucose was the most effective in restoring the activity of the substrate-depleted heart. Pyruvate was the next most effective substrate, and acetate and  $\beta$ -hydroxybutyrate were less effective. None of the other substances tested produced any recovery. Nicotinamide was ineffective in restoring the activity of the substrate-depleted heart in the presence of lactate.

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# ACTION OF A NEW PHOSPHORYLATION DERIVATIVE OF THIAMINE ON THE HEART: A STUDY OF THIAMINE TRIPHOSPHORIC ACID

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ACCORDING to clinical observations (1,2), various cardiac troubles are prominent in the symptoms of typical beri-beri, as well as of the less characterized states of B<sub>1</sub> deficiency. Confirmation of these observations has been carried out by experiments on animals experimentally deficient and exhibiting disturbances of the formation, rate, conduction and regularity of excitation (3), ectopic ventricular rhythm, and even auricular fibrillation (4). Necrotic injuries to myocardial fibers have also been described (5, 6). Falzoy (7) reckons that all cardiac deficiencies are linked to B<sub>1</sub> hypovitaminosis.

It is well known that thiamine exists in the body in its pyrophosphoric ester form, Lohmann's cocarboxylase, the rôle of which has been well studied in carbohydrate metabolism. Furthermore, Minz (8) and v. Muralt et al. (9) have demonstrated a liberation of thiamine during nervous impulse transmission. Also, the rôle of adenosine triphosphoric acid (A.T.P.) in carbohydrate metabolism, and its importance in the mechanism of muscular contraction have been demonstrated. The present investigation is directed to the effects of a new phosphorylation derivative of thiamine.

Thiamin-triphosphoric acid (T.T.P.) was recently synthesized by Velluz, Amiard and Bartos (10, 11) and studied for its coenzymatic properties. Its obvious chemical relationship to thiamin-pyrophosphate and A.T.P., and the importance of cardiac damage in the course of B<sub>1</sub> deficiency, induced us to investigate whether or not T.T.P. exerted beneficial pharmacodynamic effects on cardiac disturbances experimentally producible.

T.T.P. has only a slight toxicity; the average lethal dose for the mouse, intravenously, is approximately 0.16 mg/gm. of body weight. Through the whole course of this study, we used T.T.P. in saline, Ringer's or Tyrode's solutions at a pH of about 7.5.

We have studied the action of T.T.P. upon the following: *a*) the normal excised frog's heart and its experimental dysfunctions (12, 13); *b*) coronary output of the excised rabbit's heart (13); and *c*) fibrillation of the rabbit's heart *in situ* (14). The action of T.T.P. is compared to that of related substances, i.e., thiamine, thiamin-pyrophosphate and A.T.P.

## EXPERIMENTAL

*Excised Frog's Heart (Straub's Method)*

*Action of T.T.P. on the normal and hypodynamic heart.* At low concentrations ( $10^{-6}$  and  $10^{-5}$ ) T.T.P. induces, after a certain delay, a gradual and slight increase (from 30% to 40%) of the amplitude of beats and a slight slowing of the rate. This effect is persistent and disappears only after prolonged washing of the preparation. At higher concentrations ( $10^{-4}$  and  $10^{-3}$ ) T.T.P. has a negative inotropic effect, which may be compared to the similar action of thiamine at the same dosage (15). Cocarboxylase, at the same concentration, elicits after an initial and inconstant increase, a decrease of amplitude of the beats in a first phase and after 2 or 3 minutes augments it in a second phase.

The proper action of T.T.P. is much more marked on the fatigued heart and irregular in its amplitude and rhythm (fig. 1). After a dose of  $10^{-3}$  the heart comes back to normal beating and rhythm after a sufficient time of contact. If one replaces then the Ringer's solution containing T. T. P. by a fresh normal Ringer's solution, the heart resumes its irregular beating. Thiamin-pyrophosphate, also, will restore a regular rhythm, but is not efficient in the case of extreme fatigue, whereas T.T.P. is still active. As for thiamine itself, it has no influence on the tired heart.

*Influence of T.T.P. on the effects of chemical mediators.* When added to the preparation in Ringer's solution simultaneously with acetylcholine or epinephrine T.T.P. reduces the action of either mediator presumably by strengthening the regulating mechanisms. Jackson and Wald (15) found that the action of acetylcholine was not antagonized by thiamin-pyrophosphate though it is by vitamin B<sub>1</sub>, but only at pH above 6.

*Action on dysfunctions due to mineral ions.* One of the best known means of producing experimental cardiac disorder is by disturbing the physiological balance of ions. We realize this by addition of an excess of K or Ca ions to the medium. We have observed that, in addition to its negative inotropic effect, an excess of K<sup>+</sup> induces also some degree of block. For this action to be discernable, it is necessary to maintain the K<sup>+</sup> concentration of the solution at a correctly chosen level. The initial inotropic negative effect tends to decrease and then some periodic irregularities appear which reveal partial blocking in different parts of the conductive elements of the heart. The reactions of our preparation being thus well established, we added various amounts of T.T.P. to the Ringer's solution, either simultaneously with K<sup>+</sup>, or sometime before. In either way, we noted that, at sufficient concentrations ( $10^{-4}$  to  $3 \times 10^{-4}$ ), T.T.P. limits to a certain extent the decrease of movements caused by K<sup>+</sup>, and especially abolishes the periodic irregularities due to partial blocking (fig. 2). This effect of T.T.P. lasts a certain time even after washing. The enhancing of conduction lasts much longer than the protection from the negative inotropic effect of K<sup>+</sup>.

A.T.P. ( $2 \times 10^{-5}$ ) acts in the same way, though not as completely. Thiamine is inactive; it even increases the action of K<sup>+</sup>. Thiamin-pyrophosphate antagonizes it, but very imperfectly, even at  $10^{-3}$  concentration. Some control experiments carried out with mineral phosphates, sodium ortho-, pyro-, and triphosphate, showed that

those salts increased the amplitude of beats, but possessed no antagonistic properties towards  $K^+$ , and especially did not alter the disturbances of conduction resulting from an excess of  $K^+$ .

Another well known type of cardiac dysfunction is produced by an excess of  $Ca^{++}$  which tends to counteract the diastolic relaxation of the heart and, at high doses, stops the heart in systolic contracture. The simultaneous addition of T.T.P. ( $10^{-4}$  to  $2 \times 10^{-4}$ ) and of  $Ca^{++}$  decreases the contracture effect of the ion. At the limit concentrations of  $Ca^{++}$  which alone lead to a total arrest in systole, the beating is maintained by T.T.P., but much reduced in amplitude.  $Ca^{++}$  may sometimes cause partial blocking manifested by periodic irregularities. This is always suppressed by T.T.P. (fig. 3). In two control experiments, thiamin-pyrophosphate hardly affected the response to  $Ca^{++}$ .

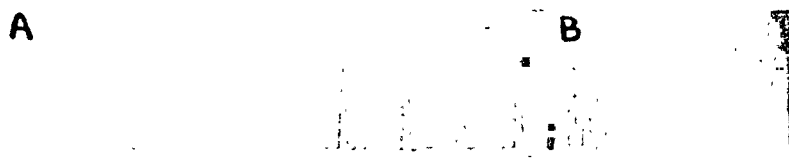


Fig. 1. EXCISED FROG'S HEART. A. Beating of a fatigued heart. B. The same heart after a 40-minute treatment with  $10^{-5}$  T.T.P.

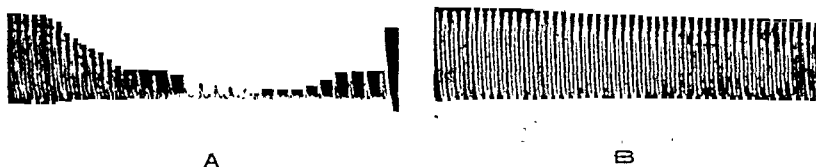


Fig. 2. EXCISED FROG'S HEART. A. Action of 0.05 mg/ml. of KCl. (in addition to that usually present in Ringer's solution). B. Action of the same dose of KCl (as in A), in presence of  $3 \times 10^{-4}$  T.T.P. in Ringer's solution.

We have also tested T.T.P. on the disorders caused in the frog's heart by  $Ba^{++}$  and have observed a notable diminution of its disturbing actions, particularly in those affecting the conduction elements. The systolic contracture due to  $0.5 \times 10^{-3}$   $Ba^{++}$  is decreased, but the most striking effect is the abolition of bigeminal rhythm (fig. 4) and other periodic irregularities.

*Action on digitoxin poisoning.* It is well known that the action of digitoxin is hardly reversible. Thus, alternate tests in the presence or absence of the substance studied are difficult. For this, we attempted to investigate the effect of T.T.P. on the functional recovery of the intoxicated heart after washing. The isolated frog's heart exhibiting typical symptoms of digitoxin intoxication, i.e. systolic contracture, and practically irreversible conduction disturbances regains gradually its normal functions after the addition of  $10^{-5}$  T.T.P. to the Ringer's bath. Signs of intoxication reappear as soon as T.T.P. is withdrawn; hence it is apparent that digitoxin is not displaced. Similar observations were made with ouabaine.

*Action on disorders induced by faradization.* Stimulation by a pair of electrodes applied to the ventricle of a frog's heart produces a complete stop of ventricular

beats followed by a period of auriculo-ventricular dissociation, preceding a return to the normal rhythm. After such stimulation in the presence of T.T.P. ( $10^{-5}$ ) there is a notable shortening of the standstill phase, as well as a total disappearance of the partial block period. The ventricle recovers immediately its initial rhythm and amplitude (fig. 5). These effects attain their maximum after a somewhat prolonged contact with the T.T.P. solution. They still persist after numerous washings of the heart. Thiamine and thiamin-pyrophosphate act likewise but a concentration 10 times stronger is required. A.T.P. occasionally reduces the standstill period, but lengthens the phase of auriculo-ventricular dissociation.

### *Isolated Rabbit's Heart*

*Effect on coronary output* (Gley's method, 16). Between the concentrations of  $2.5 \times 10^{-5}$  and  $2.5 \times 10^{-4}$ , T.T.P. increases the coronary output. The increase may be

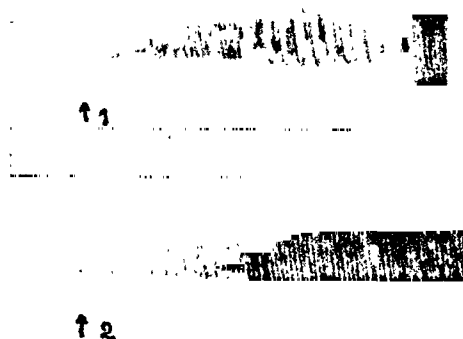


Fig. 3. EXCISED FROG'S HEART. A. Action of 2.5 mg/ml. of cryst.  $\text{CaCl}_2$  (in addition to that usually present in Ringer's solution.) B. Action of the same dose of cryst.  $\text{CaCl}_2$  in presence of  $10^{-4}$  T.T.P. in Ringer's solution.

from 50 per cent to 125 per cent and persists for 25 to 30 minutes (fig. 7). Higher concentration, on the contrary, reduces the coronary output for a period varying with the dose. The latter decrease is followed by a secondary increase corresponding to the action of the lower concentrations, the active substance tending, with the technique used, to eliminate itself from the preparation.

### *Rabbit's Heart in situ: Effect on Fibrillation*

After the experiments described above, the action of T.T.P. on fibrillation of the heart *in situ* was determined. The rabbit, anesthetized with Somnifène (0.4–0.6 mg/kg.), was given artificial respiration, the thorax was opened and the heart exposed. Fibrillation was produced by induction shocks. The electrodes were either directed to the apex of the heart at a small distance from each other, or were formed of hooks inserted, one in the right auricle and the other at the apex, for recording purposes. In that way it was possible to obtain a fibrillation either confined to the ventricle or generalized.

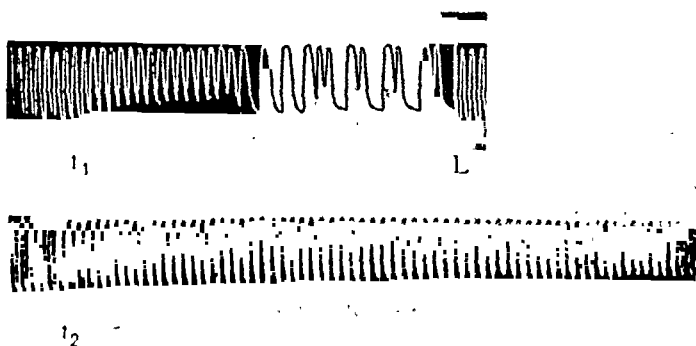
In varying the time of passage of the current and the distance between the coils, we determined the stimulus convenient to induce cardiac standstill of 30 to 60 seconds. By repeating the stimuli at regular 5- or 10-minute intervals, this functional stopping augments gradually during the control experiments. In our experiments, if the cardiac failure exceeded about one minute, the fibrillation was always fatal unless

rapid resuscitation was applied, either by direct manual massage of the heart muscle, or by intraventricular injection of KCl (10–20 mg/kg.). We often had to do both, without noticing an altering of sensitivity to further faradization.

Primarily, T.T.P. intravenously elicited only a light and fleeting hypotension even at large doses (10 mg/kg.). Its influence on the normal heart is slight, with a small increase of amplitude and slowing of rhythm. Furthermore, intraauricular or intraventricular injections of large doses (10 mg/kg.) are well tolerated.

As for fibrillation, at a dosage of 1 mg/kg. one may observe a shortening of the standstill, but the maximum effect is reached at 10 mg/kg. At that dosage, protection is seen immediately after the injection and is most powerful after a 10 minute delay. Most often there is full suppression of fibrillation; the heart resumes its normal movements as soon as the electric excitation ends (fig. 6). This protective effect lasts at least 20 minutes, and, when doses of 10 mg. are used, may persist during several hours. In some cases T.T.P. was administrated by intracardiac injection to

Fig. 4. EXCISED FROG'S HEART. A. Action of 1 mg/ml. of cryst.  $\text{BaCl}_2$  L : washing with Ringer's solution. B. Action of the same dose of  $\text{BaCl}_2$  in presence of  $10^{-4}$  T.T.P.



the fibrillating heart until it came to a standstill. The heart was seen to come back gradually to its normal beating.

These findings induced us to study the respective importance of the various parts of the T.T.P. molecule. We tested, first, some mineral phosphates—pyrophosphates and even a preparation of sodium triphosphate—in quantities equivalent to those used in the form of T.T.P.; they have a depressant effect on the heart beat and slow its rhythm, but are entirely deprived of antifibrillatory properties.

Thiamine, alone, at a dosage of 1 or 2 mg/kg. is also ineffective. At a 10 mg. dose, there is unquestionably an antifibrillatory potency, but incomplete and transient; its protective effect exerts itself immediately after the intrajugular injection by a shortening of duration of the standstill, and lasts not more than five minutes. Thiamin-pyrophosphate or 'cocarboxylase' is slightly more active in preventing fibrillation, and places itself between thiamine and T.T.P. At a 1 or 2 mg/kg. dosage, it is practically without action; larger doses (10 mg/kg.) give to the heart a relative protection of 10 to 15 minutes (with the maximum 5 minutes after injection). However, it must be pointed out that neither with cocarboxylase nor with aneurine was a full inhibition of fibrillation ever found.

#### DISCUSSION

Owing to the wide diversity of the effects of T.T.P. on the heart, the question rises of the site and mechanism of its action. In the presence of a triphosphoric

ester, the idea readily comes to mind of an action on the muscle fibers; the important part played by A.T.P. in muscular physiology is well known. The slight positive inotropic effect of T.T.P., observed after a certain time of contact, probably implies an improvement of muscle metabolism. But its immediate action exerts itself rather on the pacemaker and above all on the conduction elements. This seems to be proved by the fact that any disturbance attributable to a dysfunction of these elements is, in an immediate and spectacular way, amended by T.T.P. This is shown

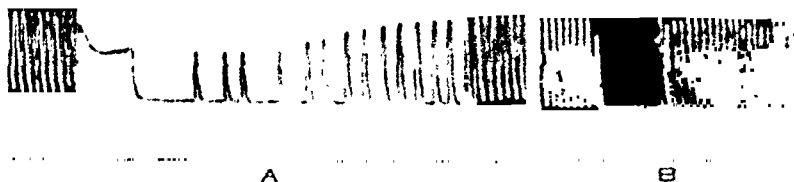


Fig. 5. EXCISED FROG'S HEART. A. Heart inhibition under faradic excitation (6 seconds with a Dubois-Raymond coil, distance between secondary and primary: 5 cm). B. Faradization under the same condition after 40 minutes contact with  $10^{-3}$  T.T.P.

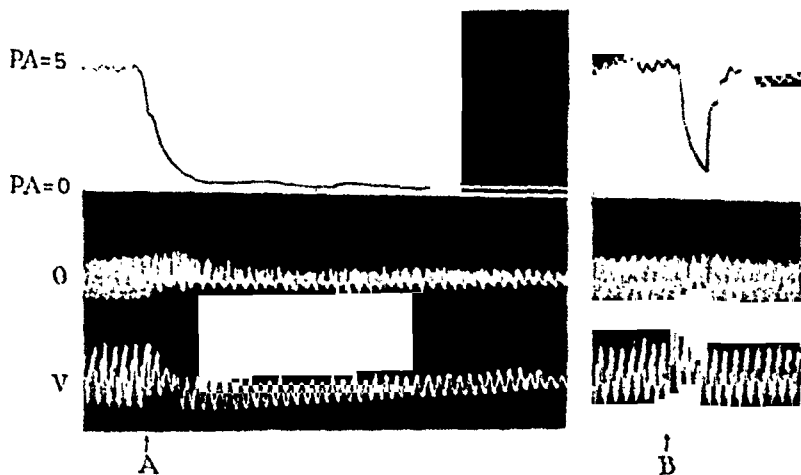


Fig. 6. RABBIT ♀, weight: 3,700 kg. Anesthesia with 'Somnifene' (0.5 ml/kg.). Artificial respiration. Heart *in situ*. From top to bottom, recording of: pressure in the carotid; auricular beats; ventricular movements. A. During 3 seconds passage of a current from an induction coil. Prolonged ventricular fibrillation. Distance between the coils: 10 cm. The heart resumed its normal functioning only after massage carried out 1 minute after stimulation. B. Same stimulus 10 minutes after an intrajugular injection of 10 mg/kg. T.T.P.: no fibrillation.

perhaps most clearly in its antagonism to  $K^+$  on the frog's heart. In the process of action of the ion one may discriminate a negative inotropic action, and periodic irregularities due to partial blocks in the different conduction elements. The influence of T.T.P., while it is unquestionable on the former abnormality, is much more remarkable on the latter. The case is the same in the majority of compounds whose actions may be explained as a strengthening of the sinusal control, presumably due largely to an improvement of conduction.

As for fibrillation, whatever the interpretation given to its intimate mechanism may be, it is generally agreed (17, 18) that its appearance is related to defects of conduction at the different levels of the heart. Thus, T.T.P., in enhancing the con-

duction of the impulse through the heart, may hinder the initiation of fibrillation and, by the same process, produce defibrillation. It seems likely that T.T.P. may interfere in the chain of biochemical reactions necessary to impose the sinusal control to the lower elements.

The heart, liver and kidneys are the three organs which contain the greatest quantity of thiamine (19), the last two acting as storehouses. On the other hand, it has been demonstrated that only a fraction of the tissue thiamine is free (20, 21), the remainder existing in the form of combinations of which only a few are known. Up to now, only one of these combinations may account for the biological importance of thiamine: its pyrophosphoric ester (Lohmann's cocarboxylase). Some authors (22, 20), however, consider that cocarboxylase is not the sole physiologically active

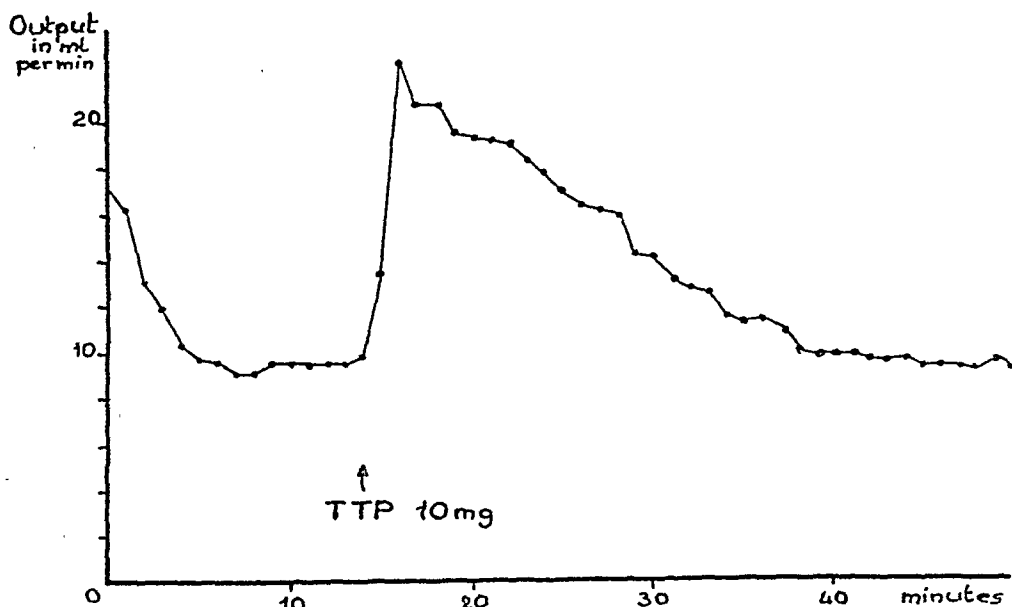


Fig. 7. EXCISED RABBIT'S HEART (Gley's method). ♂ rabbit, weight: 3,000 kg. Absc: time in minutes; ord: coronary output in ml/min.

form of thiamine. The substance designated  $A_4$  by v. Muralt (23), probably a thiamine derivative, seems to exhibit the same positive inotropic effect on the frog's heart as T.T.P. (24). Anyway, the occurrence of T.T.P. in the organism may be considered as possible and even probable if one refers to the existence of A.T.P. synthesized in the organism and, also, to the greater efficiency, as our experiments show, of T.T.P. in comparison with thiamine and thiamin-pyrophosphate.

This adds more evidence of the biological importance of the degree of phosphorylation. One can assume that the energy-rich bond of T.T.P. could supply to the nerves as well as to the conduction elements of the heart immediately available energy necessary to the transmission of the impulse, in the same way as the similar bond of A.T.P. supplies the energy necessary for the muscle contraction. Though this idea remains hypothetical, it is justifiable to consider that T.T.P. plays a rôle in the special metabolism related to nerve impulse transmission. Does it play a part in the conduction tissue of the heart, in the way of an 'ergone' as defined by v. Euler (25) or as a metabolic material, as was supposed by Liechti, v. Muralt and Reinert (26) for the thiamine in the nerve? We can, to-day, only set the problem.

## SUMMARY

The effects on the heart of a new phosphorylation derivative of thiamine, thiamin-triphosphoric acid (T.T.P.), were studied. On the excised frog's heart, T.T.P. exerts a slight positive inotropic action on the normal organ, and strengthens and restores the regularity of the fatigued heart. It decreases the action of acetylcholine and epinephrine, corrects the disturbances induced by an excess of  $\text{Ca}^{++}$ ,  $\text{K}^+$  or  $\text{Ba}^{++}$ , reestablishes the functioning of the heart intoxicated by digitoxine or ouabaine, and antagonizes the electric depression of the heart. It acts probably by improving the conduction.

On the excised rabbit's heart, T.T.P. increases the coronary output when used at concentrations varying from  $2.5 \times 10^{-4}$  to  $2.5 \times 10^{-5}$ , but at higher concentrations, it decreases the output. On the rabbit's heart *in situ*, T.T.P. protects, in an effective and prolonged way, the organ against fibrillation induced by faradization. Thiamine also exhibits some antifibrillatory properties, but weak and transient. Thiamin-pyrophosphate is more potent than thiamine but much less than T.T.P.

The problem of the existence of T.T.P. in organisms and the physiological rôle of this new derivative of thiamine is discussed.

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# TRANSFORMATION OF THE ARTERIAL PULSE WAVE BETWEEN THE AORTIC ARCH AND THE FEMORAL ARTERY<sup>1</sup>

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THERE have been many attempts to explain the observation that the arterial pressure pulse, which originates centrally as a relatively simple manifestation of cardiac ejection, appears at the periphery as a sharp primary peak of pressure followed by the prominent dicrotic oscillations of pressure. It has been proposed that an essential factor responsible for this transformation is the superposition of a standing wave upon the transmitted pulse as a result of the resonant properties of the aortic system. Definite experimental evidence has been presented by Hamilton and Dow (1) that in the abdominal aorta and femoral artery of the dog the transmitted pulses are distorted by a pressure oscillation which rises and falls simultaneously at different points of the system and which therefore must represent a standing wave.

The existence of such a standing wave and the possibility of measuring its frequency by an analysis of peripheral pulses is of considerable significance, since the period of this standing wave should be determined by the existing physical properties of the aortic system (2). Recently we employed this concept in an analysis of changes in the femoral pulse observed in hemorrhagic hypotension and shock (3). The simplifying assumption was made at that time that the peripheral pulse could be regarded as a simple fusion of the transmitted pulse with the standing wave, so that the latter could be derived by algebraically subtracting the central pulse from the peripheral pulse. This approach was of considerable value in qualitatively classifying femoral pulse types and identifying abnormal characteristics of pulses observed in shock, and therefore encouraged a more quantitative analysis with particular attention to the apparent frequency of the standing wave system.

A survey of several thousand pulse recordings, however, has convinced us that the primary peak of the femoral pulse does not correspond to the primary phase of the standing wave. The observations to be reported here serve to confirm this hypothesis and indicate that in the femoral pulse there is a significant distortion factor which in most cases masks the first phase of the standing wave.

## METHODS

Lateral pressure pulses were recorded in dogs under barbital anesthesia with optical manometers having adequate frequency characteristics. The technical details of the recording apparatus are the same as formerly described (3). Central aortic pulses were recorded from a sound introduced into the innominate artery by way of the left carotid. Lateral pressure pulses in the abdominal aorta were obtained by performing a left nephrectomy and introducing a sound into the central stump of the renal artery until its tip came flush with the wall of the aorta. Lateral

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femoral arterial pulses were recorded in a similar fashion using a short 15 gauge needle introduced into a convenient side branch of the femoral.

In order to evaluate the components incorporated into the synthesis of the peripheral pulses, the central aortic pulses were subtracted from the peripheral pulses so as to obtain what will here be referred to as the 'subtraction curve.' These curves were obtained with the coordirectograph (4), an instrument with which pulses of different pressure scales can be accurately subtracted in one simple operation. To minimize any mechanical errors the original curves were first projected and redrawn at a magnification of 2.5 times. Since high frequency vibrations in the aortic pulse such as those at the incisura are obviously not transmitted to the femoral, these were smoothed out when the curves were redrawn. The enlarged curves were then accurately lined up on the coordirectograph with the foot of the wave of both pulses at the same point on the time axis. This corrected for the transmission delay as the pulse traversed the aorta. After obtaining the subtraction curve at a scale proportional to that of the femoral pulse, the inked curves were then photographically reduced down to the same scale as that of the original recording. Subtraction curves obtained in this manner were accurately reproducible except for the initial portion where both curves rise steeply and slight mechanical errors are correspondingly exaggerated. This inaccuracy is restricted to the initial negative deflection of the subtraction curve and is of no particular importance in the problems to be considered here.

#### RESULTS

A typical curve obtained by subtracting the central aortic pulse from the femoral pulse recorded simultaneously in a normal anesthetized dog is shown in the lower portion of figure 1A. This recording was selected because at first glance the subtraction curve appears to have the sinusoidal form which is to be expected of a free oscillation that gives rise to a standing wave. More precise measurements, however, reveal that the initial positive wave does not conform to the sinusoidal contour which would be anticipated if this were the first phase of the simple oscillation represented by the following negative and positive waves.

Frequently the departure from sinusoidal contour is much more obvious than that shown in figure 1-A. Examples of such irregularities are shown in figure 1-C, D, E. Inspection of subtraction curves such as that shown in 1-E readily leads to the inference that the prominent positive peak is not related to the sinusoidal standing wave. On the contrary, what appears to be the first phase of the standing wave can be clearly seen taking off from the falling limb of this initial positive peak. The dotted lines indicate the assumed dissociation between the initial peak and the standing wave. A similar interpretation is suggested for the irregularities in figures 1-C and D.

Confirmation of this interpretation of the femoral subtraction curves is uniformly observed in the curves obtained by subtracting the central pulse from pulses recorded from the abdominal aorta. One example is given in the first cycle of figure 1-B; other examples are shown in figure 2. In this pulse the initial rise of the subtraction curve is not so prominent, and as a consequence it may be observed that in the

course of the initial positive wave a distinct second component arises. This latter component conforms with the expected frequency and contour of the initial phase of an oscillation which continues in the dicrotic waves. Since this oscillation exhibits no delay in transmission between the abdominal aorta and the femoral artery, it must represent the aortic standing wave. It therefore appears that the subtraction curve must be regarded as a composite of at least two factors; the standing wave oscillations which are dominantly responsible for the dicrotic oscillations, plus an

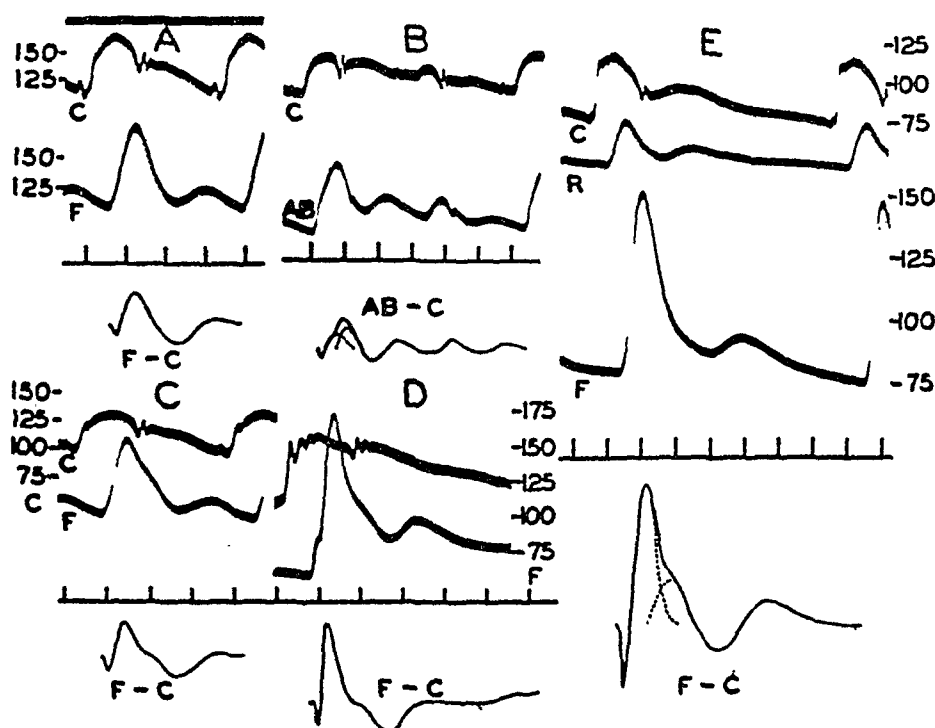


Fig. 1. ARTERIAL PRESSURE PULSES recorded from the central aorta, *C*, the abdominal aorta *AB*, the femoral artery *F*, and the radial artery *R*. Curves beneath pulse tracings were obtained by coördirectographic subtraction of the specified pulses. Dotted lines in *B* and *E* indicate the assumed dissociation between the distortion factor and the standing wave in the subtraction curve. Time intervals of 0.1 second; scales indicate pressures in mm. Hg. Reproductions about one third original size.

initial component dominating the initial peak of the pulse which we will refer to as the ‘distortion factor.’

A comprehensive treatment of the forces giving rise to the distortion factor is not within our scope at present, but a few examples selected from an extensive series of experiments will suggest the types of variation in pulse contour which may result from alterations in the distortion factor. A familiar change in pulse contour is that which is evident when lateral pressures are compared with end pressures as shown in figure 2. After recording the lateral femoral pressure shown in figure 2-A, the artery was occluded just distal to the point of cannulation so as to obtain the end pressure record reproduced in figure 2-B. The end pressure pulse recorded from the femoral exhibits additional pressure oscillations which we would interpret as due to a water hammer phenomenon, in agreement with Hamilton (5). Any such local vibrations will obviously be represented in the distortion factor as shown in the lower portion of

the figure. It should be noted that the standing wave appears to be essentially the same in both recordings; the slight variation in the abdominal subtraction curve produced by occluding the femoral artery appears to be restricted to the initial distortion factor.

Variations in stroke volume also produce marked changes in the peripheral pulse contour not directly dependent upon variations in the form of the central pulse. This is illustrated in the alternans rhythm shown in figure 1-B where the subtraction curve exhibits a very marked reduction in the distortion factor in the case of the weak cycle. These records should be compared with the pulses in figure 1-C and D. After recording the normal cycle shown in C, the heart was stopped for 2 seconds by right vagal stimulation. The large succeeding beat is shown in D. It is evident that

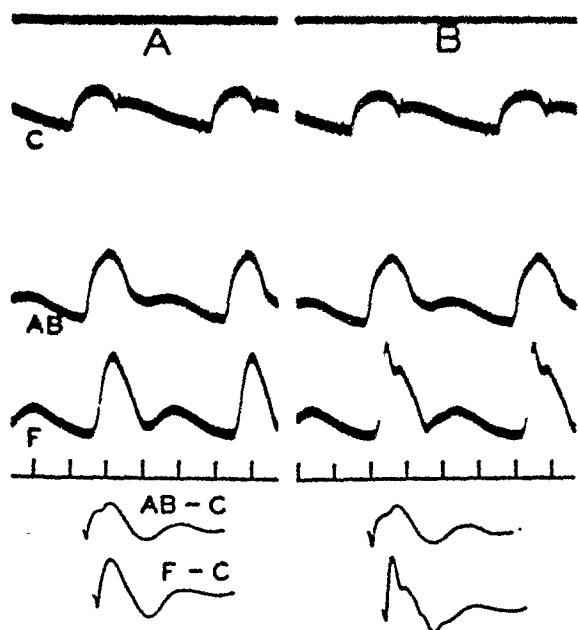


Fig. 2. NORMAL TRACING with femoral pressure recorded laterally, A, compared with pulses observed after occluding the femoral just below the point of cannulation so as to record end pressure, B. Symbols etc. the same as in fig. 1:

the major change in the contour of the femoral pulse is due to the great augmentation of the distortion factor with a large stroke volume.

#### DISCUSSION

The present study offers additional support for the standing wave concept. An extensive series of simultaneous recordings of abdominal aortic and femoral pulses have uniformly confirmed Hamilton and Dow's contention that these pulses contain an oscillating wave which rises and falls simultaneously at different points of the system. Furthermore, situations in which the apparent form of this standing wave were not in keeping with predictions may now be recognized as due to a confusion of the distortion factor with the standing wave. For example, the fact that the primary peaks of pulses recorded from the aortic-femoral system do not always occur simultaneously can no longer be used as an argument against the standing wave theory.

Recognition of the importance of the added distortion factor in the femoral pulse contour is of considerable significance from the standpoint of evaluating the standing wave. The primary peak of the femoral pulse is dominated by the distor-

tion factor and in many pulses this distortion factor also encroaches considerably upon the dicrotic notch. Since neither of these points can be used reliably to identify the standing wave, the period of this standing wave cannot be accurately measured by analysis of the femoral pulse contour. Because of comparable difficulties in other pulses, we question whether any pulse can be used routinely to obtain an accurate estimate of the standing wave frequency.<sup>2</sup>

On the basis of the known properties of the aortic system, the distortion factor would appear to result from the interaction of several forces. Most obvious is the distortion which results from simple damping of the transmitted wave. For example, the initial upward deflection of the central pulse produced by the early phase of cardiac ejection usually represents a pressure change of too high frequency to be transmitted along the aorta. This is shown by the fact that the ascent of the foot of peripheral pulses usually exhibits a somewhat more gradual slope. This damping is revealed in the subtraction curves by the initial negative deflection preceding the major positive wave. In addition, it is obvious that high frequency vibrations in the central pulse at the incisura together with similar vibrations sometimes appearing on the anacrotic limb are also damped out of the transmitted pulse. Damping of the central pulse in its transmission towards the periphery therefore accounts for some distortion, but quantitatively it can account for only a small part of the distortion factor observed in the femoral pulses recorded at reasonable pressure levels.

Closely related to damping are properties of the aortic system which distort the pulse wave by altering the transmission rate of its successive portions. Pulse wave velocity increases as a function of the pressure existing in the aorta. Thus if the central aortic pressure rises steeply enough, portions of the pulse wave initiated at significantly higher pressures than the foot may tend to overtake the foot of the wave and result in a greater peaking of the femoral pulse (6). In the extreme form this results in pulses of the water hammer type as will be discussed in a subsequent communication (7). Acting opposite to this factor is the hysteresis in the aortic system (2, 8), as a consequence of which the aorta becomes relatively more rigid at the end of diastole than it is after being subjected to some degree of systolic stretch. This would tend to accelerate the transmission of the foot of the wave as compared with later portions of the pulse. These properties acting directly to change the contour of the pulse wave in its transmission will result in distortions which would be incorporated into the curve obtained by subtracting the central pulse from the peripheral pulse.

Finally, it appears probable that local reflections make a significant contribution to the distortion factor. This is obviously true in the case of end pressures such as that shown in figure 2-B where reflections from the occluded end of the vessel generate

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<sup>2</sup> In employing the subtraction curve method, it is essential to correct for the pulse transmission delay if the transmitted component is to be correctly dissociated from other factors in the peripheral pulse. However, since the standing wave makes some contribution to the contour of the central aortic pulse, the method used here leads to a corresponding error in the magnitude and some phase distortion in the derived standing wave component of the peripheral pulse. This error is not of sufficient magnitude to invalidate the qualitative recognition of the components in the subtraction curve because the aortic pulse is recorded from a point much nearer the node of the standing wave than is the femoral pulse. Nevertheless this would represent an additional source of error in attempting to quantitate the standing wave by this method.

a water hammer oscillation. Since the vascular bed represents essentially a closed system with resistance increasing progressively toward the periphery, one should expect positive reflections of the transmitted wave to occur generally throughout the system. Indeed, it is assumed that the summated effect of these reflections in the aortic-femoral system as a whole is responsible for the genesis of the standing wave. It therefore would appear that any pulse recorded from a given local region must also be somewhat distorted by reflections from its various local tributaries.

According to this analysis, therefore, the femoral pulse may be regarded as a transmission of the central aortic pulse summated with the standing wave oscillation, together with a significant degree of distortion resulting from various physical properties of the aortic system. It is unfortunate that the complexity of these distorting forces makes it difficult to assess the forces responsible for any given pulse contour. For example, the fact that increased stroke volume greatly augments the distortion factor could be explained by a corresponding increase in reflections. On the other hand, it could also be due to a greater peaking of the pulse in its transmission because of the large pressure difference between the foot and the summit of the wave. Until it is possible to define these variables with greater qualitative accuracy, attempts to subject the femoral pulse to any rigorous quantitative analysis (9) are, in our opinion, decidedly premature.

In conclusion, it is of interest to review our previous interpretation of the pulse contour in shock (3) in the light of these present observations. At that time a peculiar distortion of the subtraction curve in shock was interpreted as possibly due to the summation of two standing waves of different frequency. It now appears that the sharp initial peak of the femoral pulse in shock should be attributed to the persistence of an essentially normal distortion factor rather than to a high frequency standing wave component. On the other hand, the present observations serve to strengthen the assumption that the altered catacrotic limb and dicrotic waves of the shock pulse are due to the appearance of a standing wave of low frequency and amplitude. Whether this can be attributed to a specific dilation of the mesenteric bed, as suggested previously, remains speculative.

#### SUMMARY

Arterial pressure pulses recorded optically in anesthetized dogs have been analyzed by subtracting the central arterial pulse from pulses recorded from the abdominal aorta and the femoral artery so as to obtain the 'subtraction curve.' The subtraction curves obtained from the femoral arterial pulses do not usually conform with the simple sinusoidal pattern that would be expected if they represented solely the oscillation of the aortic standing wave. Under certain conditions, however, a definitely sinusoidal oscillation appears in the subtraction curve in spite of the presence of a significant 'distortion factor' that usually dominates the primary peak of the femoral pulse curve.

Analysis of lateral pulses from the abdominal aorta demonstrates a similar distortion factor followed by a standing wave oscillation which can be clearly identified. The magnitude of the distortion factor varies as a direct function of the central pulse pressure. It exhibits additional components in end pressures as compared with

lateral pressures. Probable sources of the distortion factor are discussed. They include damping of the transmitted wave, distortion of this wave by variations in pulse wave velocity, and local reflections.

These observations serve to reinforce the concept that the femoral arterial pulse contains a prominent pressure oscillation due to a standing wave created in the central aortic system, but indicate the necessity for distinguishing the first phase of this oscillation from the wave representing the distortion factor.

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# ARTERIAL PULSE DYNAMICS IN AORTIC INSUFFICIENCY<sup>1</sup>

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SINCE its original description in 1832, there has been considerable speculation as to the factors responsible for the 'Corrigan' pulse observed in the peripheral arteries of patients with insufficient aortic valves. The simplest explanation has been that its rapid rise and sudden collapse directly parallels the forceful ejection of a distended heart followed by the regurgitation of blood through the incompetent valves. The impossibility of such an interpretation was first recognized by Stewart (1) who called attention to the fact that a significant portion of the collapse of the peripheral pulse occurs during the latter part of systole while the heart is still ejecting blood. Recognition of this paradox has prompted the invocation of a variety of mechanisms to explain the pulse dynamics, particularly some sort of unique vaso-motor response to alter the peripheral dissipation of pressure (1).

The problem is further complicated by a lack of agreement as to the detailed contour that is observed in such pulses. In dogs with experimental aortic insufficiency, femoral pulses recorded directly with optical manometers reveal a simple sharp rise and fall in pressure with a significant reduction in the dicrotic waves (2). On the other hand, Feil and Gilder (3), who made an intensive sphygmographic study of patients with aortic insufficiency, found the frequent occurrence of pulses with double peaks (bisferiens). Other authors have claimed that there is an accentuation of the dicrotic waves in aortic insufficiency. Luisada (4) believes that the second murmur associated with such pulses (Duroziez's sign) is directly related to an accentuated dicrotism.

Although some of these discrepancies might be related to instrumental artifacts which are difficult to exclude in the sphygmographic techniques employed in clinical studies, it must also be remembered that clinical aortic insufficiency is not a simple syndrome. In rheumatic valvular disease, for example, aortic insufficiency is typically accompanied by a significant degree of aortic stenosis. In addition, progressive compensatory changes in chronic valvular disease such as the marked hypertrophy of the left ventricle might readily introduce alterations in pulse dynamics which did not relate directly to an incompetency of the aortic valves. To clarify the fundamental dynamics of the arterial pulse in aortic insufficiency, it, therefore, seems necessary to investigate this problem in acute experiments where the immediate changes produced by a known lesion may be studied.

## METHODS

This analysis is based upon optical recordings of the aortic, radial and femoral pulses in dogs under barbitol anesthesia. The techniques employed in obtaining these pulses by direct cannulation of the respective vessels were the same as those previously described (5, 6). To permit direct comparison of normal pulses and pulses in the presence of the lesion, aortic insufficiency was produced acutely and reversibly without damage to the valves. This was accomplished by opening the chest in the mid-line and introducing through the wall of the left ventricle either a large trocar

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which when passed into the aortic orifice served to hold the valve leaflets open (7) or an umbrella-type valve spreader with which any degree of insufficiency could be produced at will (8). The damage to a local area of the myocardium and the mild degree of functional stenosis induced by both of these procedures produced only minor changes in cardiac dynamics as judged by the central pulse contours. The peripheral pulses obtained by this method were quite comparable to those obtained from dogs with chronic aortic insufficiency (2).

#### EXPERIMENTAL RESULTS

In figure 1 *A* and *B* is shown the typical change produced in the aortic, radial and femoral pulses by an insufficiency of mild degree. The initial cycles of figure 1 *C* and *D* illustrate the changes observed with an essentially complete absence of valve func-

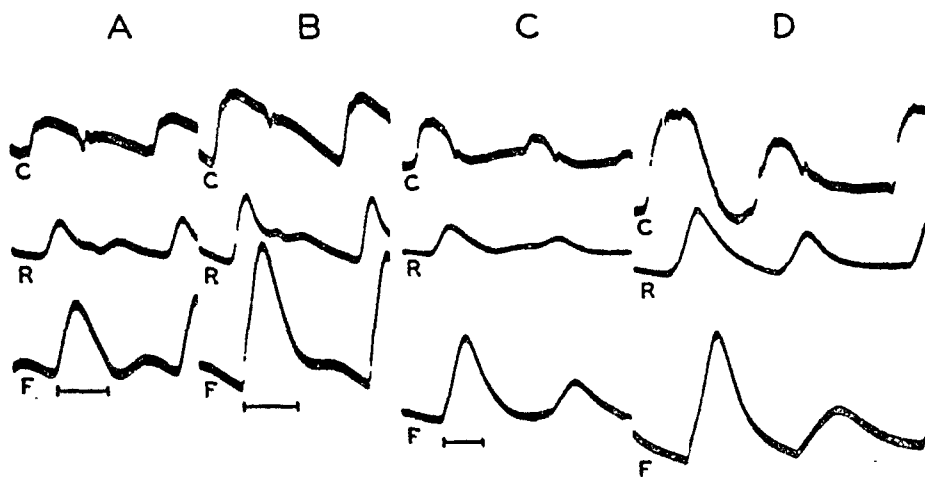


Fig. 1. OPTICAL RECORDINGS of the pressure pulses in the central aorta, *C*, the radial artery, *R* and the femoral artery, *F*. *A* and *C* were recorded before the production of aortic insufficiency; *B* and *D* immediately afterwards. Irregularity of beats in *C* and *D* due to arrhythmia associated with manipulation of the instrument. Bars beneath femoral pulses indicate duration of systole. Time signal indicates 0.1 second.

tion. The progressive changes in contour obtained in successive recordings by a progressive increase in the degree of incompetency are shown in figure 2.

The most obvious characteristic of the peripheral pulses during aortic insufficiency is the rapid rise to a very high peak pressure, yielding a very large pulse pressure. Can this extremely high peak be explained in terms of our basic concepts regarding the genesis of the peripheral pulse? It has been shown that the peak of the femoral pulse is dominated by a distortion factor (5) which normally augments the systolic peak of the femoral pulse as compared with the central aortic pulse. To ascertain whether there is anything unique in this augmentation of the systolic peak in aortic insufficiency, it becomes important to compare pulse pressure changes observed in a variety of other conditions. Such data are given in table 1 in which pulse pressures recorded with identical techniques but under a variety of experimental conditions are compared. To indicate the relative changes in the femoral pulse pressures, they have been expressed as a ratio to the central aortic pulse pressure, as indicated in the right hand column. Although there are marked changes in pulse

pressure in all of these instances, it will be noted that the relative changes between femoral pulse pressure and central aortic pulse pressure are slight. In the first five experiments listed, the only significant changes in this ratio are the reduction in the case of the alternans rhythm and the increase produced by adrenalin, both of which pulse changes have previously been discussed (5, 6). In typical cases of experimental arterio-venous fistula, however, there is a tendency for the relative increase in the femoral pulse pressure to fall in spite of a large increase in the absolute femoral pulse pressure. A similar situation holds for the two typical cases of aortic insufficiency in which the relative increase in the femoral pulse pressure falls in spite of an actual increase in femoral pulse pressure of well over 100 mm. Hg, and femoral systolic peak pressures that are over 50 mm. higher than the central aortic systolic pressure. These data make it quite clear that no special assumptions are required to

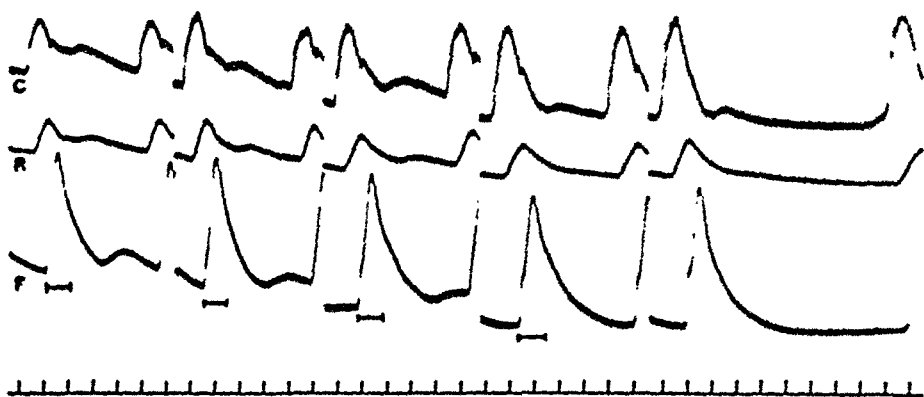


Fig. 2. PULSE RECORDINGS during the progressive production of complete aortic insufficiency about four cycles have been omitted between each cycle shown. The last cycle was recorded during a vagal bradycardia.

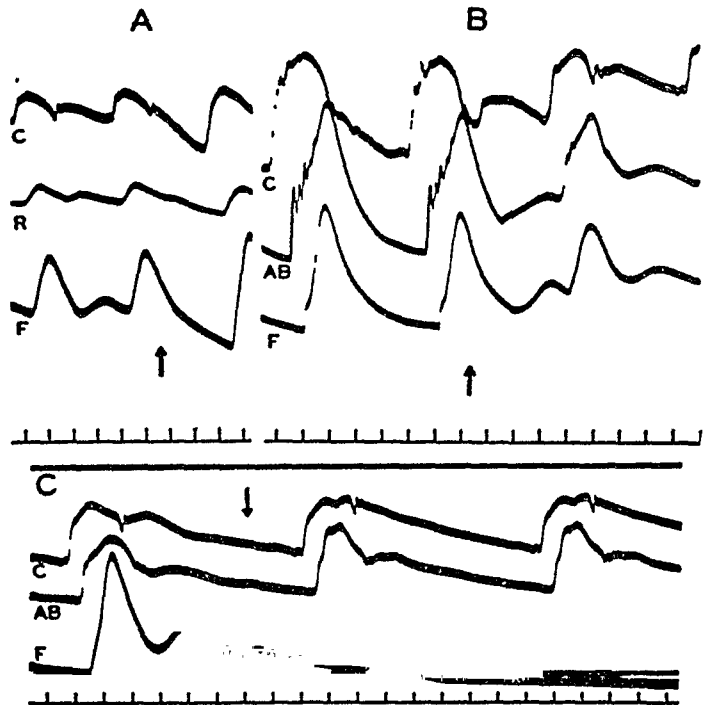
explain the great magnitude of the peripheral pulse pressure in aortic insufficiency. It is merely a consequence of the high aortic pulse pressures together with the distorting forces which normally lead to a significant augmentation of the peak pressure in peripheral pulses. The fact that the pulse pressure ratio actually drops somewhat in this case is probably related to the decline in mean blood pressure that is usually observed in acute experimental lesions.

When the rise in pressure in the femoral artery is studied in greater detail, two further points of interest come to light. First, it should be noted that although in the normal case the initial rise of the femoral pulse is not as steep as that of the central pulse (fig. 1A), in the case of aortic insufficiency the initial rise of the femoral pulse usually is as steep or steeper than the pressure rise in the aorta. In the normal case, the more gradual ascent of the femoral pulse appears to be due essentially to damping of the transmitted pulse wave (5). This indicates that in the case of aortic insufficiency there must be some other distorting force which counteracts this damping process.

The nature of this force is suggested by a second characteristic of the femoral pulse in aortic insufficiency: the existence of a 'halt' in the pressure rise part way up

the ascending limb as shown in figures 2 and 3*B*. When this halt is analyzed by the subtraction curve method (5) it is found to be a high frequency vibration superimposed upon the steep pressure rise, an initial positive phase followed by a negative phase acting to initially accelerate and then retard the rise in pressure. It has long been recognized that in aortic insufficiency conditions exist for the production of water hammer phenomena as the steep pulse wave front is transmitted peripherally to vascular beds which are at a low diastolic pressure and therefore relatively stagnant. A water hammer oscillation of this type seems to be the major characteristic of the ascending limb of the Corrigan pulse. It should be emphasized, however,

Fig. 3. PULSE RECORDINGS as in previous figures; *B* and *C* include a pulse recorded from the abdominal aorta, *AB*. As described in the text, arrows indicate: *A*, production of the lesion; *B*, removal of the lesion; *C*, point of compression of the abdominal aorta at the bifurcation of the iliacs. No aortic lesion in *C*.



that this water hammer should not be confused with the high systolic peak of pressure to which it is often incorrectly ascribed. As discussed above, the augmentation of the peak of pressure in the femoral artery is not dependent upon this water hammer effect. It should also be noted that the water hammer oscillation represented by a halt in the ascending limb of the femoral pulse is not unique with aortic insufficiency, but has been observed in a wide variety of other conditions in which there was a high systolic discharge coupled with a low diastolic pressure.

Turning to the descending limb of the pulse, inspection of the pulses in figures 1 and 2 in which the duration of systole has been indicated just beneath the femoral pulse affords ample confirmation of the fact that the major collapse of the Corrigan pulse occurs before the completion of cardiac systole when correction is made for the delay in transmission of the pulse wave to the periphery. The collapse therefore cannot be explained directly on the basis of regurgitation through incompetent valves. A fact that is too little appreciated, however, is that this 'systolic collapse' of the peripheral pulse is in no way unique in aortic insufficiency. Such a relationship is typical of normal peripheral pulses as indicated in figures 1*A* and 2*A*. As has been

explained previously (5), this relationship is due to the fact that the peak of the femoral pulse is not determined directly by the contour of the transmitted pulse wave but by a significant distortion factor which may be regarded as being superimposed upon the transmitted wave. It has been pointed out above (cf. table 1) that in aortic insufficiency the magnitude of this distortion factor is greatly increased in absolute though not in relative terms.

Viewed in this light, the steep fall of the catacrotic limb of the femoral pulse in aortic insufficiency can best be described as stated by Wiggers (9): "It requires no profound knowledge of physics to understand that the higher the rise, the greater must be the collapse which follows." Actual measurements of this collapse in six

TABLE 1

EXPERIMENTAL CONDITION	SYS/DIAS. CENTRAL	CENTRAL PULSE FREQ.	SYS/DIAS. FER.	FEM. PULSE PRESSURE	FEM. PULSE AREA CENTRAL PULSE FALL.
Trachea occluded	108/76	32	127/76	51	1.59
Extreme resp. var.	137/68	71	173/66	107	1.51
Normal cycle	148/130	18	169/118	51	2.83
Alternans cycle	139/132	7	140/124	16	2.29
Normal control	163/138	25	187/135	52	2.68
Following vent. premature	167/124	43	212/117	95	2.21
Normal control	148/120	28	179/118	61	2.18
Vagal bradycardia	140/86	54	201/87	114	2.12
Normal control	163/113	50	205/110	95	1.90
After adrenalin	216/124	92	316/120	196	2.13
Normal control	140/98	42	186/87	99	2.36
Expt. A-V fistula	142/73	69	212/60	152	2.22
Normal control	126/91	35	157/89	68	1.94
Aortic insuff.	135/52	83	183/43	140	1.69
Normal control	140/120	20	176/119	57	2.85
Aortic insuff.	151/80	71	204/30	174	2.45

different experiments, moreover, reveal that although the gradient of fall is steeper than normal, the *relative* rate of fall is slower than normal. This can be illustrated by measuring the time required for the pressure to fall half way from its systolic peak to the diastolic level. As shown in table 2, this interval for the fall to the half-way point is significantly longer in all but one of the pulses recorded with aortic insufficiency as compared with the control pulses recorded immediately before production of the lesion. This indicates that in relative terms the Corrigan pulse should not be considered a 'collapsing' pulse but rather a pulse which falls relatively slower than normal.

The explanation for this relatively slower fall obviously resides in the depression or absence of the dicrotic waves. The Corrigan pulse does not fall steeply into the dicrotic notch to subsequently rebound into the positive dicrotic wave as in the normal pulse, but exhibits a relatively smooth gradient from the systolic peak to the end of diastole. To prove that there were no delayed dicrotic waves submerged in

the succeeding pulse, the heart was slowed by right vagal stimulation in obtaining the last record shown in figure 2, where the complete lack of dicrotic waves is clear. In milder cases of insufficiency the dicrotic waves persist but with a reduced amplitude as shown in figure 1*B*. This change in the dicrotic waves applied to both the femoral pulse tracings and the radial pulse tracings.

It has been shown that such a reduction in the dicrotic waves accompanies vasodilation (10). The instantaneous nature of this change, however, excludes the possibility of such an explanation here, as shown in figure 3*A* and *B*. These two recordings were selected because of the fortunate timing of the production and the removal of the insufficiency. In figure 3*A* it will be noted that the insufficiency was produced at such a time that the pulse shows no change until the end of the incisura. There is then a moderately steep fall of the diastolic portion of the central pulse indicating a moderate degree of insufficiency. The accompanying femoral pulse which exhibited a perfectly normal primary wave exhibits a corresponding fall free of the definite dicrotic waves evident in the preceding cycle. The radial pulse shows comparable changes although a small dicrotic wave still persists. In a similar fashion, restoration of the valve competency shortly after the end of systole (fig. 3*B*) produces

TABLE 2

PULSE	PULSE PRESSURE MM. HG		TIME FOR HALF FALL, SEC.	
	Control	Insufficient	Control	Insufficient
Radial	45	67	.049	.059
	59	114	.038	.061
Femoral	48	85	.057	.073
	71	116	.060	.062
	44	91	.055	.077
	40	124	.039	.044

an abrupt rise in the central pulse and the appearance of a delayed but prominent dicrotic wave in the femoral pulse.

Remembering that the dicrotic waves are essentially a manifestation of the aortic standing wave (5, 11), it, therefore, follows that incompetency of the aortic valves abolishes this standing wave. This might prompt one to return to the older view that the dicrotic waves are simply a rebound phenomenon against the aortic valves independent of the peripheral vascular tree, and therefore the dicrotic waves would be a peripheral manifestation of the central incisura. Reaffirmation of the importance of the peripheral vascular bed in the genesis of these dicrotic waves is shown in figure 3*C*. The initial cycle of this recording shows normal central, abdominal aortic, and femoral pulses. The prominent dicrotic waves in the femoral pulse are paralleled by similar dicrotic waves of reduced magnitude in the pulse recorded from the abdominal aorta. In the central aortic pulse the standing wave may be identified as pressure oscillations opposite in phase to the peripheral dicrotic waves. At the point indicated by the arrow, the abdominal aorta was occluded at the bifurcation into the iliac arteries. This not only prevents the pulse from reaching the femoral, but it will be observed that it produces a marked change in the dicrotic or standing wave oscillations in both the central aortic and the abdominal aortic pulses. Analysis of these

pulses by the subtraction curve method (5) reveals, as would be expected, a significant shortening of the wave length of the standing wave.

It is, therefore, evident that the standing wave giving rise to the dicrotic oscillations is correctly visualized as originating in the periphery by what is basically a reflection phenomenon. Nevertheless, the aortic system must be considered as an essentially closed resonator in which both ends of the system must be maintained intact in order to set the system into resonant oscillation. Opening the central end of this resonator by aortic insufficiency acts to reduce its resonance in much the same fashion as the creation of an 'opening' at the periphery by intense vasodilation.

It follows that although failure of the aortic valves to close should destroy this resonance, it should not abolish the primary reflection process at the periphery. In other words, if our interpretation of the standing wave is correct, the central aortic pulse should still exhibit evidence of the initial reflection arriving back from the periphery even though the standing wave oscillation does not arise. Actual evidence of such a reflection is always clearly discernible in the central pulse tracings in severe insufficiency provided the heart rate is slow enough to dissociate successive cycles. This is illustrated in the right hand recording of figure 2 by the small but definite pulsation shortly after the systolic wave of the central pulse tracing.

A final characteristic of the Corrigan pulse which warrants particular emphasis is the high degree of damping which is observed in the transmission of the pulse wave. This is partly suggested by the low magnitude of the reflected wave which has just been described. Still clearer examples of this damping process are given in figure 1C and D. These recordings were obtained from one of the few dogs which fairly consistently showed cardiac irregularities with slight manipulation of the instrument used to render the valves incompetent. In figure 1C the valves are functioning normally but manipulation induced the weak second cycle shown in the figure. A directly comparable event occurred a few moments later after the instrument was in place to prevent closure of the valves (fig. 1D). Comparison of these central pulses and the accompanying femoral pulses makes it clear that gross damping of the pulse wave occurs in aortic insufficiency. The greater damping of the weak pulse in aortic insufficiency is particularly well brought out when it is observed that the central pulse pressure in this weak cycle in 1D is actually greater than the pulse pressure of the initial normal beat in 1C.

Two factors contributing to this increased damping are the low diastolic blood pressure in aortic insufficiency as well as the high to and fro velocity of blood flow in this condition (12). This damping would further serve to smooth out the contour of the peripheral pulses and unfortunately it has been found to invalidate attempts to accurately analyze Corrigan pulses by the subtraction curve method (5).

#### DISCUSSION

In a previous analysis (5) the arterial pulse has been considered as the algebraic summation of a transmitted pulse wave, a significant distortion factor that dominates the primary peak, and the aortic standing wave which is responsible for the dicrotic waves. The present study indicates that femoral pulses observed in acute experimental aortic insufficiency may be readily explained in terms of these basic principles

without invoking any unique mechanism to account for the pulse changes observed. Since the pulses recorded in these acute experiments portray the essential features of the typical femoral pulse observed in clinical cases of aortic insufficiency, the same basic dynamics would appear to be involved in both instances.

The dynamics of the normal radial pulse have not been studied as intensively as in the case of the femoral pulse, but there is every reason for assuming that the same fundamental principles apply to both pulses. The only major difference between these two pulses is that the brachio-radial system is not a part of the central aortic resonator but has a standing wave system of its own (10). Nevertheless, in theory such a system should be influenced by aortic insufficiency in the same manner as the aortic-femoral system. The actual recordings of radial pulse changes reproduced in the foregoing figures are quite in accordance with this prediction.

It will be noted that in the present study we have never observed pulses with the complex double peaks described by Feil and Gilder (3). Unlike the defect produced here, however, rheumatic valvular disease typically produces a significant degree of stenosis accompanying aortic insufficiency. Katz *et al.* (13) have shown that with aortic stenosis there is a sharp initial rise in pressure in the central aorta followed by a more gradual rise to a summit late in systole. This ejection pattern superimposed upon the dynamic alterations due to aortic insufficiency could readily result in exaggerated water hammer effects so as to yield pulses of the bisferiens type. We would, therefore, agree with the general interpretation proposed by Feil and Gilder that their various pulse types of the water hammer, anacrotic, and bisferiens classes were essentially different forms of the same basic process. In addition, it might be noted that the sphygmographic technique employed in clinical studies must inevitably lead to some compression of the artery at the point from which the recording is obtained, a process which in itself may contribute to oscillations of the water hammer type (5).

In regard to the degree of dicrotism of the pulses, it follows from our argument that any significant degree of aortic insufficiency must inevitably be accompanied by a reduction in the magnitude of the standing wave oscillation responsible for the dicrotic waves. This does not imply that there must be a complete absence of a dicrotic wave in the typical clinical case comparable to that observed in extreme experimental aortic insufficiency. In the second and third pulses in figure 2 it will be noted that some dicrotic wave persists even though the central pulses indicate a definite valvular insufficiency. In chronic lesions in which the defect had been more fully compensated, an even greater persistence of the dicrotic wave is to be anticipated. Nevertheless, there appears to be no sound theoretical or factual basis for Luisada's contention that in aortic insufficiency there is an accentuated dicrotism (4). Actually his own published figures as well as the pulse tracings published by others offer no support for such a viewpoint. The change in pulse form observed by Luisada with occlusion of the artery would appear to have quite a different explanation (5). Therefore, since the diminution of the dicrotic oscillations reduces the tendency for the pulses to fall sharply into the dicrotic notch, it is apparent that the term 'collapsing' is highly inaccurate as a characterization of the Corrigan pulse. This conclusion is in accord with the suggestion made by Feil and Gilder (3) that the collapsing quality 'is more imagined than real.'

## SUMMARY

Insufficiency of the aortic valves was produced acutely in anesthetized dogs while recording with optical manometers the femoral arterial pulse together with the simultaneous central aortic pulse, and in some instances the radial pulse. Analysis of the pulse pressures obtained in these recordings, when compared with pulse pressure changes obtained under a variety of other conditions, indicate that there is nothing unusual about the peripheral pulse pressures in aortic insufficiency; the large values observed are merely a consequence of the large central pulse pressure together with the relative augmentation of this pulse pressure that is observed in normal peripheral pulses.

A water hammer oscillation is responsible for the sharp upstroke and irregularities on the ascending limb of peripheral pulses in aortic insufficiency, although the water hammer does not produce the high systolic peak of pressure. The catacrotic limb of the peripheral pulse in severe aortic regurgitation is characterized by an absence of the dicrotic waves. This is due to a loss of the resonant properties of the aortic system which give rise to the aortic standing wave. The present study gives added support to this standing-wave concept.

In relative terms the Corrigan pulse does not fall as rapidly as the normal pulse since it does not exhibit the dip into the dicrotic notch. It therefore cannot be accurately characterized as a 'collapsing' pulse.

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# OBSERVATIONS ON THE HEPATIC VENOUS CIRCULATION WITH SPECIAL REFERENCE TO THE SPHINCTERIC MECHANISM

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IT HAS been demonstrated by Graña, Mann and Essex (1) that the intravenous injection into dogs of a number of substances, such as extracts of *Ascaris suum* and hydatid cyst fluid, caused a marked engorgement of the liver whether *in vivo* or *in vitro*. In an intact animal the hepatic congestion was accompanied by severe shock as evidenced by an extreme decrease in arterial blood pressure and death of the animal if the dose of the substance was sufficiently large. That the cause of death resided in the liver was shown by the fact that hepatectomized dogs were little affected by doses that had proved lethal for dogs possessing their livers.

The present report is the result of an investigation of the possible hepatic vascular mechanism by which the findings just mentioned were obtained.

Numerous studies of the physiology of the hepatic blood vessels have been made. Bauer and his associates (2) have reviewed the earlier work which recognized that blood flow through the liver depends partially on intrahepatic mechanisms. Mautner and Pick (3) postulated that the hepatic veins might serve as a 'throttle' mechanism. They described a 'nervous mechanism' located in the end capillaries of the portal vein or the beginning capillaries of the hepatic vein or in between, which resulted in spasm of the capillaries when activated by injections of peptone or histamine or by an anaphylactic response. Subsequently, Mautner and Pick (4), and numerous other workers, have elaborated and modified their original idea but the 'throttle' mechanism as a basic physiologic concept persists. The modifications of the idea of a throttle mechanism have been concerned with its exact location within the liver, the species variation in its operation, and pharmacologic responses of the mechanisms.

An early recognition of the anatomic variations in the hepatic veins was made by Brissaud and Sabourin (5), who described valves formed from folds of the vessel wall and reinforced by muscle fibers. These were present in the dog and seal. Mall (6) noted a difference in the portal and hepatic veins and described the normal structure of the hepatic veins as being a 'spiral valve,' in the dog and cat. Gilbert and Villaret (7) doubted that the structures were valves and described them as 'furrows' or simple folds in the walls of the subhepatic veins. This term was used in contradistinction to valves. All of these were purely anatomic observations. The structures were considered normal hepatic anatomy and not until Mautner and Pick's (3) observations concerning the effect of 'shock poison' on the hepatic veins specifically was the difference in the anatomy of the portal and hepatic veins considered physiologically significant. Subsequently numerous anatomic and physiologic studies have been made on the so-called throttle or sphincteric mechanism.

Several significant observations have been made on the anatomy of the hepatic veins since their importance was recognized. Arey and Simonds (8) showed that the hepatic veins of dogs contained a relatively enormous amount of smooth muscle in comparison with the hepatic veins of the other mammals studied.

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Elias and Feller (9) described a constriction protruding into the lumen at the caval end of each hepatic vein in freshly obtained human livers fixed with hot (70° to 80° C.) formalin. Miyake (10) stated that the hepatic veins of the human, in contrast with the portal venous branches, are equipped with a rather thick coat of longitudinally disposed muscle fibers. Popper (11) made a rather complete anatomic study of the hepatic vessels and concluded likewise that dogs had relatively large amounts of smooth muscle in the hepatic veins. He also studied veins fixed under the influence of histamine and found almost complete closure. Man had relatively less smooth muscle, but Popper expressed the belief that there was some evidence of a throttle action where smaller radicles opened into larger veins.

Arey (12) reported that after studying 30 mammalian species, only 3, the dog, raccoon and seal, showed peculiar arrangements of muscle in the hepatic venous tributaries. He described the dog's hepatic veins as containing longitudinal smooth muscle in the larger tributaries; and in the central and sublobular veins it assumes shapes which may be interpreted as rings or spirals.

Deysach (13) described a new anatomic structure to help account for the 'throttle' mechanism. In a study of the cat and rabbit particularly but including various other animals (raccoon, Virginia opossum, grizzly bear, white-tailed deer and others), he described what he called 'small sluice channels.' These small sluice channels are peculiar side branches of the sublobular veins which arise from the confluence of many sinusoidal capillaries. They penetrate more or less perpendicularly through all of the tunics of the sublobular veins. Normally, in Deysach's opinion, these small sluice channels are open and a greater or lesser amount of blood from the sinusoidal capillaries by-passes the central veins and flows directly into the sublobular veins. When the throttle mechanism is activated, the constriction of the sublobular veins closes the ostia of these small sluice channels, forcing all the blood to pass through the central veins, which he termed, for purposes of comparison, 'large sluice channels.' These, Deysach stated, are also narrowed by the contraction of the sublobular veins but are not obliterated as are the small sluice channels. He studied the effect of various drugs on these small sluice channels. Epinephrine and atropine relaxed the sublobular veins and opened the small sluice channels, but mecholyl chloride and pilocarpine closed them.

Snyder (14) reviewed the literature on the anatomy of the hepatic veins, emphasizing the reviews and discussions of this subject by Pfuhl and Tischendorf. He stated that the veins of the livers of many vertebrates have a peculiar distribution of smooth muscle. In hepatic veins and venules of dogs and amphibious mammals, "the smooth muscle cells are not arranged in continuous sheets as in ordinary veins, but grouped into slender spirally arranged bands, and especially where the hepatic venules empty into the hepatic vein, the band becomes thickened and quite annular, so as to provide a sphincter." He stated that humans have circular and longitudinal sheets of smooth muscle in the hepatic veins as well as annular sphincters at the vena caval ostia which serve in a manner similar to sphincters of the alimentary canal.

Du Mais (15) studied the normal and pathologic vasculature of the liver by injection of neoprene latex to make corrosion casts. He described a series of ringlike constrictions throughout the entire hepatic vasculature inclusive of the central veins. This was most marked in the hepatic veins of the dog. These were less prominent, but present, in the rat. Du Mais also described rings of contraction in the portal vein and venules which were less pronounced than in the hepatic veins. Similarly he described a characteristic constriction pattern in the hepatic artery. He stated that the segments were much closer to each other than in the veins but the constrictive folds were intermediate in size between those of the hepatic and portal veins.

Weatherford (16) studied the histologic anatomy of the livers of animals in anaphylactic shock. In addition to great increases (up to  $8\frac{1}{2}$  times) in lymph flow and a marked fall in arterial blood pressure he observed certain microscopic changes in the hepatic tissues. He noted an initial swelling of the hepatic cells, followed later by 'cloudy swelling,' hydrops, vacuolization of the cytoplasm and central necrosis of the cells. Later still he observed stasis of the blood in the narrowed distal sinusoids which led to formation of hyaline plugs or thrombi.

The previous pharmacologic studies divide themselves into those designed 1) to determine the location and 2) to determine the nature of the sphincteric mechanism.

One of the early studies was that of Mautner and Pick (3), in which the effects of anaphylactic shock, peptone shock and histamine shock were investigated and the physiologic mechanism of the shock was found to be in the liver.

Simonds and Brandes (17) agreed that the mechanism of peptone shock was in the liver, and they further localized it in the hepatic veins. They found that livers of shocked animals showed a marked decrease in blood outflow. Also, if the liver was placed in an airtight chamber and its volume was measured accurately, the volume increased when perfused into the portal vein and decreased when perfused into the hepatic vein, showing that the block was on the hepatic venous rather than the portal venous side of the central vein.

Mautner and Pick (4) likewise made studies of the changes in volume produced by histamine to confirm their original impression that the sphincteric mechanism was on the hepatic venous side. They localized the mechanism in the smaller tributaries.

Bauer, Dale, Poulsson and Richards (2) studied the effects of various doses of epinephrine and histamine. Their conclusion was that the sphincteric mechanism was located near the caval openings of the hepatic veins of the dog, and that the tone of the vascular tissues was increased by histamine and relaxed by small doses of epinephrine. They further added that there was less complete evidence that the constrictor effect of histamine extends with diminishing effect on the deeper part of the hepatic veins and their branches. They felt that the liver of the cat and the goat did not show definite evidence of a constrictor mechanism.

As already stated, Graña, Mann and Essex showed conclusively that the sphincteric mechanism when activated by injections of extracts of *Ascaris suum*, was in the liver and in the hepatic veins.

In addition to these pharmacologic efforts to locate the mechanism, other experiments have been done to determine what drugs cause relaxation or constriction of the mechanism. Snyder concluded that cholinergic drugs caused a contraction of the mechanism, and that adrenergic drugs have either little or no effect on the mechanism.

Waters and Markowitz (18) showed that typical anaphylactic shock could be obtained in liverless dogs. This contradicted the earlier opinions that the sphincteric mechanism was the essential factor in canine anaphylaxis. They did not deny, however, that in the dog with a liver, the liver was an important influence in the production of shock.

Tainter and Dock (19), Wollheim (20), Katz, Rodbard, Friend and Rottersman (21), Katz and Rodbard (22) and others showed that digitalis in appropriate doses increased the portal pressure and reduced blood volume through activation of the sphincteric mechanism of the hepatic veins.

There has been some conjecture as to the purpose of the sphincteric mechanism. Arey pointed out that the activity of the sphincteric mechanism has been emphasized in anaphylactic shock. This is probably a rare phenomenon in the dog in nature. He suggested that the alternate relaxation and contraction of the smooth muscle of the hepatic veins might be a method of aiding circulation in the normal animal.

Lamson and Roca (23) approached the usefulness of the mechanism from a somewhat different angle. They pointed out that by constriction of the sphincteric mechanism, the portal pressure, and therefore the filtration pressure, is increased. The liver can handle large amounts of fluid, and in this way can remove fluid from the circulation without the fluid's being lost from the body.

Up to the present none of these hypotheses of the location or purpose of the sphincteric mechanism has been universally accepted.

#### METHODS

The observations reported in this paper were made on 30 dogs, 5 cats, 6 rabbits and 8 rats. All animals were anesthetized with pentobarbital sodium.

In all dogs the carotid artery was cannulated and attached to a mercury manometer. The portal vein and systemic venous pressures were obtained by the insertion of polythene tubing into the vein after the method of Hoffbauer, Bollman and Grindlay (24) and attachment of the tubing to water manometers. Enough heparin was used to prevent the clotting of blood in the manometer system.

In the first 8 experiments the location of the tip of the tubing used for measuring portal venous pressure was varied between the splenic vein, a large mesenteric vein, the portal vein and as far as 9 cm. within the substance of the liver by way of the

portal vein. Variations in the position of the cannula within these limits did not vary the responses; consequently in all subsequent experiments the portal vein was cannulated by way of a small branch of the splenic vein. The tip of the tubing was then threaded through the portal system into the portal vein itself so that it lay between the last tributary branch and the liver itself.

Similarly the systemic venous pressures were measured at various levels including the jugular, superior vena cava, inferior vena cava, hepatic vein and up to 8 cm. within the liver itself by way of an hepatic vein. These various positions of the tubing did not alter the character or degree of change in the venous pressure. Consequently in subsequent experiments, the vein most convenient in that particular animal was used. Frequently it was the jugular vein with the tip of the tubing passed into the superior vena cava. The procedures followed when cats were used were the same as described for the dog.

In the experiments with rabbits, only arterial pressure was measured. In the experiments with rats, pressure measurements were not taken.

The *Ascaris* extract was prepared as described by Machéboeuf and Mandoul (25).

In the observations of the effects of anaphylaxis on the hepatic circulation the dogs were sensitized by alternate intravenous and subcutaneous injections of 1 cc. of horse serum given daily for 6 days. Anaphylaxis was induced in the third week by the intravenous administration of 5 cc. of horse serum.

The material used for the injection mass was vinyl acetate (Ward Biological Co. Rochester, New York). It was injected from dry syringes and a dry needle with the use of a large glass syringe. Because of its rapid hardening, it was injected as speedily as possible. One hundred cubic centimeters of the vinyl acetate were injected in most of the livers in order to compare more effectively the hepatic circulation of controls and treated animals.

Smaller amounts of vinyl acetate were used for the rat, rabbit and cat. The injections were usually done immediately after ligation of the vena cava above and below the liver. In the controls this was done, when possible, when blood pressure and all conditions were normal. The ligations were done rapidly and the injections were made immediately thereafter.

Injections were made into the hepatic artery and the portal vein and in a retrograde manner into the hepatic veins. In several livers injections were made into the portal as well as the hepatic vein.

After injection, the livers were allowed to remain *in situ* from 10 to 30 minutes. They were then removed and placed in glass jars containing concentrated hydrochloric acid for the digestion of the hepatic substance. The time required for this depended on the relative quantity of hydrochloric acid to hepatic substance and varied from several hours to several days. It could be hastened by the addition of relatively large amounts of concentrated acid. The specimens were then washed in water and examined under the dissecting microscope. Neutralization of the acid residue by washing in soda solution has proved advantageous.

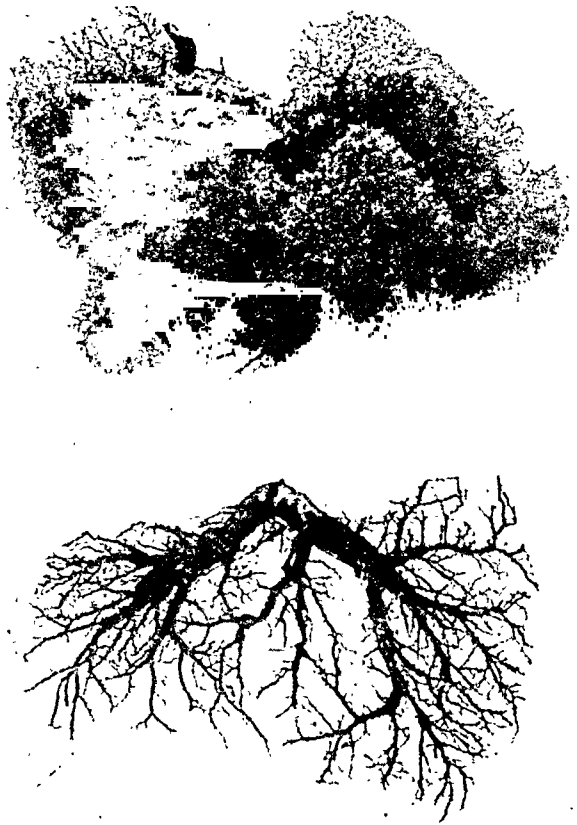
## RESULTS

*Controls.* In the control dogs, the injection mass filled the veins of the livers when injected from the portal as well as the hepatic venous side. The finest trib-

utaries, excluding the sinusoids themselves, were usually filled. There were occasional rings of constriction observed in the hepatic veins but these were relatively few and appeared to be shallow. No rings of constriction were observed on the portal side.

*Ascaris Suum Extract.* The injection of 5 cc. of a saline extract of *Ascaris suum* caused the intensive response previously described by Graña, Mann and Essex. The liver became turgid and purplish—almost black—it increased 50 to 150 per cent in size, and its surface exuded sanguineous fluid. Smears of this fluid showed the presence of many red cells. The portal pressure rose rapidly and to dramatic heights.

Fig. 1. *A. (upper).* CAST OF HEPATIC VEINS of a control dog. *B. (lower).* Cast of the hepatic veins after an injection of an extract of *Ascaris suum*. This cast was indistinguishable from those made after anaphylactic or histamine shock had been induced ( $\frac{1}{2}$  natural size).



On one occasion it rose from 7 to 31 cm. of water. The usual rise was 10 to 20 cm. of water above the control pressure taken before administration of the drug. Thus it is seen that the increase in portal pressure was from 70 to 300 per cent. The systemic venous pressures fell from 2 to 6 cm. during the rise in portal venous pressure. This occurred whether or not the tubing was passed up to 8 cm. within the substance of the liver, which was, therefore, well beyond the junction of the hepatic veins and the vena cava. The carotid pressure, as measured by a mercury manometer, decreased depending on the dosage of *Ascaris* extract, from an initial value of 100 to 170 down to 40 to 0 mm. of mercury. Five cubic centimeters of the crude extract as an initial dose was practically always fatal, but as little as 0.05 cc. for each kilogram of body weight in a few experiments caused all the foregoing effects and the death of the dog.

An examination of the cast of the veins of the liver showed no unusual effect on the portal system, but the changes in the hepatic venous system were dramatic and very remarkable. Grossly the cast of these vessels appeared to be made up only of large vessels because many of the smaller branches did not fill. On inspection of the cast with the aid of a dissecting microscope, it was evident that there was an intense spasm of the entire hepatic venous vasculature. This spasm took the form of bands of constriction arranged in a spiral fashion, which frequently gave the vessels the appearance of a corkscrew. This was more pronounced in the smaller vessels, but occurred definitely also in many of the larger ones. In some instances the constriction appeared to be more pronounced at the junction of two vessels. The effects of extracts of *Ascaris suum* were also marked on four isolated perfused livers of dogs (figs. 1 and 2).



Fig. 2. PERIPHERAL PORTION of an hepatic vein showing the spiral form of the smaller branches

*Anaphylaxis.* Two of the 4 dogs were insufficiently sensitized and a drop in carotid pressure or any other signs of anaphylaxis did not follow the injection of the horse serum. There was not a change in portal venous pressure or in the gross appearance of the liver. Consequently the livers of these dogs were not injected. After the injection of the antigen the other 2 animals showed a typical anaphylactic reaction with a sharp decrease in carotid artery blood pressure. In the first dog the portal pressure rose from 8 to 25 cm. of water and the carotid blood pressure fell from 160 to 10 mm. of Hg. The liver was injected in a retrograde manner, and the cast of the vessels showed the typical corkscrew-like pattern. The second dog showed a similar reaction (figs. 1 and 2).

*Histamine.* Four dogs were injected with 0.01 mg. of histamine base per kilogram of body weight. A response of the hepatic vessels similar to that described for anaphylaxis was obtained. Two of the livers of dogs that received 1 mg. of histamine were injected with vinyl acetate and showed the typical rings of constriction.

*Anoxia.* In one dog, the hepatic artery was ligated. After 2 hours, the liver was injected with vinyl acetate. The cast of the hepatic veins showed the typical rings of constriction.

*Cats.* Five cats were injected with extracts of *Ascaris suum*. The hepatic circulation of 2 showed the typical response. The livers of the cats did not show the dark color seen in the livers of dogs. Two gave an atypical response, one of these showed a rise in arterial blood pressure. Casts of the vessels of the livers that responded showed contraction rings which were much less pronounced than in the dog.

*Rabbits.* Observations were made on 4 rabbits. The carotid blood pressure decreased after injections of extracts of *Ascaris suum*. Casts of the blood vessels of the liver were not greatly different from those of the controls.

*Rats.* Eight rats were used. Three were used as controls. Five were given injections of *Ascaris* extract. The effects on the liver were questionable. Constriction rings could be seen in the casts of the hepatic vessels, but they were less pronounced than those seen in the casts of the hepatic circulation of dogs.

#### SUMMARY AND CONCLUSIONS

The existence of a sphincteric or 'throttle' mechanism in the liver of the dog has long been appreciated. Largely on a histologic anatomic basis it was considered unique to dogs. Two principal ideas as to its exact location have been put forward. Mautner and Pick (3, 4) localized it in the smaller hepatic veins. Bauer and his associates presented evidence that it was a muscle ring at the entrance of the hepatic veins into the vena cava. This was based on a physiologic and anatomic conception. This latter view has gained most acceptance. We first accumulated evidence that there was not a marked constriction in that part of the hepatic vein by passing a polythene catheter up to 9 cm. within the liver, well beyond any single sphincter, and finding pressure readings typical of the systemic venous pressure rather than the portal venous pressure following injections of appropriate drugs.

This study has shown that the mechanism is, in fact, a diffuse spasm of the entire hepatic venous side of the vasculature of the liver. This is even more pronounced in the smaller vessels than in the larger ones.

This spasm may be induced in dogs by extracts of *Ascaris suum*, hydatid cyst fluid, anaphylactic shock, histamine, digitoxin and probably by protamine. It has also been observed after the hepatic artery had been occluded for 2 hours. These constriction rings were not present or were extremely slight in the livers of several control dogs.

The hepatic venous spasm has been produced in some but not all cats, and in white rats, but insufficient data were obtained to draw final conclusions in this regard in species other than the dog.

Accurate localization of this mechanism should open new areas for investigation. It is obvious that observers in the past have looked for a different type of mechanism. There is some evidence in this study that other animals show the phenomenon.

There is slight suggestive evidence that this mechanism may be present in man. This is listed as follows: 1) the well-known shock caused by spillage of hydatid cyst fluid into the peritoneal cavity, which may be caused by a spasm of the hepatic

venous system; 2) certain cases of Chiari's syndrome in which a closure of hepatic veins is discovered at necropsy; 3) the much-disputed reduction in circulating blood volume on digitalization of a patient, which may be owing to an increased resistance of the hepatic veins resulting in storage of blood in the liver.

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# HYPOPROTHROMBINEMIA DUE TO LOSS OF INTESTINAL LYMPH

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**D**URING investigations (1, 2) conducted on rats which had complete intestinal lymph fistulas, it was noted that clotting of the lymph in the cannulas rarely occurred after the first eighteen hours of the experiment. Animals which had lost lymph, moreover, sometimes bled excessively from very minor abrasions, at times bled spontaneously into the cannula, and were more than usually likely to have pericardial hemorrhage after puncture of the heart. The present study was instituted to investigate this tendency to bleed.

## METHODS

The experimental animals were male albino rats of the Sprague-Dawley strain weighing 190 to 220 gm. They were fed a standard commercial ration prior to operation and a balanced diet *ad lib*, or by stomach tube, after operation. The operative technics employed and the cages in which the animals were maintained have been described previously (3, 4). Specimens of blood were obtained by puncture of the heart, with the animal under ether anesthesia, at the various times indicated (tables 1 to 4). Specimens of lymph were collected in Erlenmeyer flasks containing 5 cc. of tenth-molar solution of sodium oxalate. Prothrombin assays were made by a slight modification of the two-stage procedure (5). The vitamin K preparation used was 4-amino-2-methyl-1-naphthol (as the hydrochloride). One milligram of this was given subcutaneously.

## RESULTS

Seventeen normal rats studied in this series had an average plasma prothrombin concentration of 336 units per cubic centimeter with normal values ranging from 270 to 400 units per cubic centimeter.

In animals which had lost all their intestinal lymph marked hypoprothrombinemia developed (table 1). This developed within eighteen hours after lymphatic cannulation and persisted as long as free flow of lymph continued. Vitamin K given parenterally prevented the development of this hypoprothrombinemia (table 1) and corrected it completely after it had developed even with continued loss of lymph (table 2).

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One animal (table 3), given vitamin K, was able to correct its hypoprothrombinemia and to maintain its plasma prothrombin at normal levels, even though it was bled excessively on two successive days and even though it lost appreciable quantities of prothrombin in the lymph.

The intravenous injection of large quantities of fresh rat plasma into animals with intestinal lymph fistulas gave an immediate partial restoration of the concentration of plasma prothrombin, but 24 hours later, hypoprothrombinemia was again extreme (table 4). In 1 animal the intravenous injection of 15 cc. of fresh rat plasma on two successive days likewise failed to prevent the development of hypoprothrombinemia.

TABLE 1. HYPOPROTHROMBINEMIA DUE TO LOSS OF INTESTINAL LYMPH: ITS PREVENTION BY VITAMIN K

RATS GIVEN	HR. AFTER CANNULATION	PLASMA PROTHROMBIN U/CC.
No vitamin K	18	43
	24	31
	45	< 10
	46	< 10
	46	16
	48	< 10
	48	23
	70	28
	72	< 10
	94	23
Vitamin K	48	270
	70	400

TABLE 2. A STRIKING RESPONSE TO VITAMIN K (RAT E<sub>2</sub>, WEIGHT 240 GM.)

HOURS SINCE CANNULATION	TOTAL LYMPH cc.	PROTHROMBIN		
		In lymph Units per cc.	Total	In plasma Units per cc.
24 <sup>1</sup>	27	16	430	16
48	42	57	2,400	400

<sup>1</sup> 2 cc. blood drawn, then 1 mg. vitamin K injected.

#### DISCUSSION

Hypoprothrombinemia has been produced in the rat by other methods (6-9) The complete loss of intestinal lymph produced it both promptly and to a profound degree. One animal with an hepatic lymph fistula, on the contrary, gave a good flow of lymph for 43 hours without development of hypoprothrombinemia. It is important to emphasize not only this specificity of the intestinal lymph in producing this effect, but also the fact that *all* the intestinal lymph had to be drained externally or else no change occurred in the blood. Attempts to correct the hypoprothrombinemia by reinjecting the collected lymph, however, proved unsuccessful because of toxic manifestations.

The amount of prothrombin actually lost in the intestinal lymph is relatively small and most of the decrease of the plasma prothrombin must be due to its (nor-

mal) utilization and lack of formation. It would appear that all of the vitamin K absorbed from the intestine enters the body by way of the intestinal lymph. The intestinal lymph, in contrast to lymph from other parts of the body, contains considerably more fat and phospholipids than does the plasma even after long periods of fasting. Fat-soluble vitamin K, formed by intestinal bacteria, is being supplied to the body during fasting.

The rapidity with which hypoprothrombinemia develops under the conditions of these experiments, shows that, in the rat, very little vitamin K is stored, that vitamin K is continually in demand to permit prothrombin synthesis, and that prothrombin reserves are small. Since large quantities of plasma fail to maintain the

TABLE 3. MAINTENANCE OF THE LEVEL OF PLASMA PROTHROMBIN WITH VITAMIN K, DESPITE LOSS OF BLOOD AND INTESTINAL LYMPH (RAT E<sub>1</sub>, WEIGHT 220 GM.)

HOURS SINCE CANNULATION	TOTAL LYMPH cc.	PROTHROMBIN		
		In lymph	Total	In plasma
		Units per cc.		Units per cc
24 <sup>1</sup>	62	27	1,670	110
48 <sup>1</sup>	41	27	1,110	340
72 <sup>2</sup>	55	14	770	340

<sup>1</sup> 4.5 cc. of blood drawn, then 1 mg. vitamin K injected.

<sup>2</sup> 4.5 cc. of blood drawn; 5.6 gm. of hemoglobin per 100 cc.

TABLE 4. EFFECT OF INJECTION OF PLASMA

TIME	PLASMA PROTHROMBIN	
	Rat I 62	Rat I 63
	Units per cc.	
Immediately prior to injection.	13	20
Immediately after injection	175	165
1 hour after injection	175	175
24 hours after injection	55	19

Twenty-four hours after cannulation of intestinal lymph vessels 10 cc. of fresh rat plasma containing 340 U prothrombin cc. was injected intravenously into each of 2 rats. Blood specimens were drawn at stated times.

prothrombin concentration, it seems likely that plasma contains little vitamin K and inadequate prothrombin to supply the animal's requirements for longer than a few hours. The ability of the rat to maintain an adequate concentration of prothrombin despite marked loss of blood and lymph shows that, if vitamin K is given, prothrombin can be formed despite depletion of protein.

#### SUMMARY

When all of the intestinal lymph of the rat was drained externally, marked hypoprothrombinemia, as determined by the two-stage method, developed rapidly, usually within 24 hours. If adequate amounts of vitamin K were administered parenterally, a normal level of prothrombin was maintained, despite loss of lymph and even of considerable amounts of blood as well. Transfusion of twice the animal's normal volume of plasma did not maintain a normal value for prothrombin while lymph was

lost. Under the conditions of these experiments it appeared that vitamin K was absorbed practically exclusively through the lymph and very little of it was stored, while the turnover of prothrombin was extremely rapid.

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# EFFECT OF ASCORBIC ACID, D-ISOASCORBIC ACID AND GLUCOASCORBIC ACID ON COBALT POLYCYTHEMIA IN THE RAT AND RABBIT

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**B**ARRON and Barron (1) have demonstrated that the simultaneous administration of ascorbic acid with a cobalt salt to rabbits prevented the development of polycythemia. If their animals were allowed to develop the polycythemia, then the intravenous administration of 60 mg. of ascorbic acid per day resulted in a decrease in both the red cell count and the hemoglobin content. Davis (2) found that the oral administration of 8 mg/kg. of ascorbic acid to dogs reduced the polycythemia resulting from the administration of cobaltous chloride. On the other hand Chen (3) reported that supplementing the diet of mice with ascorbic acid increased the red cell count and hemoglobin concentration. No cobalt was used in his work with mice.

The present study was initiated in order to determine whether ascorbic acid had a similar effect in the polycythemic rat as that reported in the case of the dog and rabbit. Also, an attempt was made to determine whether the reported effect was due to the ascorbic acid *per se* or whether compounds with similar structures but without the Vitamin C activity could alter the course of cobalt polycythemia in the rat and the rabbit.

## METHODS

Polycythemia was produced in hooded rats by the daily administration of cobaltous chloride subcutaneously at a level of 1 mg. per day, 6 days a week. This is essentially the method of Orten (4). The animals received the cobalt injections during the entire study. The diet consisted entirely of Purina dog chow. When the elevated hemoglobin concentration appeared stable for at least two weeks, the various substances to be tested were administered either orally or intravenously. The oral feedings were accomplished by the use of a rigid stomach tube. All intravenous injections were made in the tail veins. When rabbits were used the animals were rendered polycythemic by the subcutaneous administration of 10 mg. of cobalt chloride per day in the form of a 1 per cent solution (1). The rabbits were fed Purina rabbit chow supplemented by cabbage twice a week. After the animal became polycythemic the ascorbic acid and its derivatives were given intravenously into one of the marginal ear veins. All animals were still receiving the cobalt when the test acids were injected.

Hemoglobin concentrations were estimated by the acid hematin method with the final readings being made in a photoelectric colorimeter. The instrument was calibrated with human blood whose oxygen capacity had been determined.

Ascorbic, D-isoascorbic, and D-glucoascorbic acids<sup>1</sup> were tested for their possible effect on cobalt polycythemia. In the case of the rats, ascorbic and isoascorbic acids were given at two levels, 25 or 50 mg. per day. Glucoascorbic acid was administered at a level of 32 mg. per day, an amount equimolar to 25 mg. of ascorbic acid. Solutions to be given intravenously were neutralized with the theoretical amount of  $\text{NaHCO}_3$  immediately before their administration. The rabbits all received the 50 mg. dose of ascorbic and isoascorbic acids and a 64 mg. dose of the glucoascorbic acids.

TABLE 1. EFFECT OF ASCORBIC, ISOASCORBIC, AND GLUCOASCORBIC ACIDS ON THE HEMOGLOBIN CONCENTRATION OF THE BLOOD OF THE RAT

SUPPLEMENT	DOSE	ROUTE	NO. OF ANIMALS	HEMOGLOBIN		
				Before supplement	Weeks after supplement	
					1	2
	mg/day					
Control, no cobalt.....	0		15	$14.1 \pm 0.2^1$	$14.3 \pm 0.3^1$	$14.1 \pm 0.1^1$
Control, plus cobalt.....	0		9	$17.9 \pm 0.2$	$17.8 \pm 0.3$	$18.4 \pm 0.2$
Ascorbic acid.....	50	Orally	4	$18.2 \pm 0.7$	$17.8 \pm 0.6$	$18.3 \pm 0.5$
Ascorbic acid.....	25	Orally	5	$17.8 \pm 0.4$	$17.9 \pm 0.3$	$17.8 \pm 0.6$
Isoascorbic acid.....	50	Orally	5	$17.1 \pm 0.1$	$17.2 \pm 0.7$	$17.5 \pm 0.7$
Isoascorbic acid.....	25	Orally	5	$18.4 \pm 0.3$	$17.0 \pm 0.3$	$17.3 \pm 0.5$
Glucoascorbic acid.....	32	Orally	5	$17.7 \pm 0.4$	$18.1 \pm 0.3$	$17.4 \pm 0.7$
Ascorbic acid as sodium salt.....	25	I. V.	11	$18.4 \pm 0.3$	$17.8 \pm 0.3$	$18.1 \pm 0.5$
Isoascorbic acid as sodium salt.....	25	I. V.	6	$17.8 \pm 0.2$	$17.8 \pm 0.4$	$17.7 \pm 0.4$
Glucoascorbic acid as sodium salt.....	32	I. V.	8	$17.4 \pm 0.3$	$17.5 \pm 0.3$	$17.1 \pm 0.4$

<sup>1</sup> Standard error =  $\pm \sqrt{\Sigma d^2/N(N-1)}$ .

## RESULTS

The first group of rats received orally 50 mg. per day of either free ascorbic or isoascorbic acids for a two-week period without producing any effect on the hemoglobin level of the blood. From these data it appears that neither orally administered ascorbic nor isoascorbic acids had any effect on the polycythemia produced by cobalt. The data are summarized in table 1.

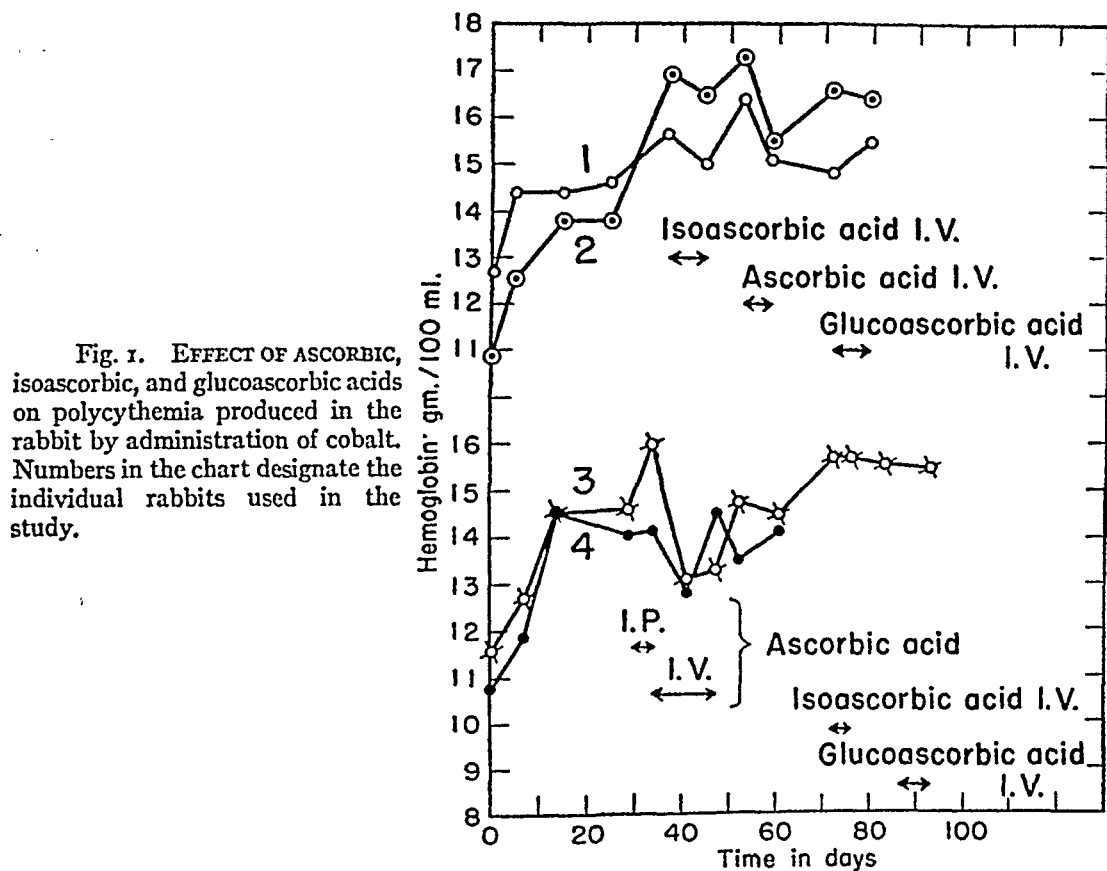
In the remainder of the study on rats smaller doses of the various acids were used because attempts were made to administer the test substances on an equimolar basis, and free glucoascorbic acid possesses a limiting solubility. The oral administration of ascorbic or isoascorbic acid at 25 mg. per day for two weeks did not in-

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<sup>1</sup> These compounds were kindly supplied by Charles Pfizer and Company, New York.

fluence the hemoglobin concentration of the blood of rats receiving cobalt. Gluco-ascorbic acid also was without effect when it was administered at an equimolar level. When similar concentrations of these three acids were neutralized with an equivalent amount of  $\text{NaHCO}_3$  and injected intravenously in rats no effect was observed on the polycythemia during the two week test period.

In the rabbit glucoascorbic acid intravenously administered had no general effect on the polycythemia produced by cobalt. Two of the three animals tested showed a fall in hemoglobin concentration following the injection of isoascorbic acid (fig. 1).



Ascorbic acid caused a marked decrease in the hemoglobin concentration in all of the animals tested. In one rabbit, however, there was an increase in hemoglobin following the initial fall, while the animal was still receiving the ascorbic acid. It may be concluded that ascorbic acid reduces the increased hemoglobin concentration produced by cobalt in the rabbit. Isoascorbic acid also does this but to a lesser extent. This is summarized in figure 1.

#### DISCUSSION

From the data presented it appears that free ascorbic acid when given orally or its sodium salt when administered intravenously does not reduce the polycythemia produced by cobalt in the rat. Isoascorbic and glucoascorbic acids were also without effect on the polycythemia. On the other hand, intravenously administered ascorbic

acid produced a marked drop in the hemoglobin concentration of rabbits made polycythemic by cobalt. Isoascorbic acid may also reduce the hemoglobin concentration in these animals but to a smaller extent. The action of the ascorbic acid in reducing the polycythemia in the rabbit is in agreement with the work of Barron and Barron (1).

From this limited study it appears that there is a parallelism between the antiscorbutic power of the compounds tested and their ability to reduce the hemoglobin concentration of polycythemic rabbits. Ascorbic acid caused the greatest drop in hemoglobin concentration and is also the most potent antiscorbutic agent. Isoascorbic acid caused a smaller drop in the hemoglobin concentration. This acid has been reported to possess only one twentieth the biological activity of ascorbic acid (5). Glucoascorbic acid did not cause any change in the cobalt induced polycythemia. The latter compound has been reported to possess only one-hundredth the antiscorbutic power of ascorbic acid (5). In fact, Woolley and Krampitz (6) have reported the production of a condition which they believed to resemble vitamin C deficiency in both cotton rats and mice by feeding glucoascorbic acid at levels of 10 per cent of the diet.

#### SUMMARY

Polycythemia was produced in rats and rabbits by subcutaneous daily injections of cobalt chloride. The oral administration of either ascorbic, isoascorbic or glucoascorbic acid to the rat did not influence the polycythemia. Likewise, the intravenous administration of the sodium salts of either ascorbic, isoascorbic, or glucoascorbic acid did not have any effect on the polycythemia in the rat. The intravenous administration of the sodium salt of ascorbic acid caused a marked decrease in the hemoglobin concentration of the blood of polycythemic rabbits. Isoascorbic acid, as the sodium salt, produced a similar decrease but of a smaller magnitude. Glucoascorbic acid was without effect on the polycythemia of the rabbit.

Acknowledgement is made to Dorothy L. DeZelia and Simon Kalish for aid during this work.

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# REACTIVITY OF BLOOD VESSELS IN THE SYMPATHECTOMIZED HUMAN LEG

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**S**ECTION of the sympathetic nervous system in animals results not only in signs of vasoconstrictor release but also in an increase in the vascular response to humoral factors, particularly adrenalin. This response is best brought out by determining the minimal effective dose. In man not only is the phenomenon of sensitization still controversial (1, 2) but there is added confusion due to the fact that in the studies mentioned, adrenalin was administered by the intravenous route and changes in surface temperature were used as a gauge of skin circulation.

Recently (3) it was pointed out that sympathetic denervation of the human limb led to an increased blood flow to the skin; in a muscular area like the calf or forearm, the blood flow remained relatively unchanged. Heating, muscular exercise or periods of vascular occlusion followed by release produced substantial increases in muscle blood flow. The conclusion was that the local metabolic needs played the dominant role in determining blood flow in muscle.

In another paper (4) it was shown that adrenalin injected into the brachial artery in the normal forearm had a dual effect upon blood flow. With fractions of a gamma the flow increased; with doses of several gamma it was diminished. That this was not dependent upon cardiovascular responses could be seen from the absence of blood pressure or pulse rate changes or other manifestation of generalized sympathetic activity. It was thought that the blood flow changes under these conditions were local vascular phenomena.

In the present study, vascular sensitivity and sensitization in the human subject were investigated by the injection of adrenalin into the main artery of a sympathectomized lower extremity. Observations were made of the effect on the blood flow to skin and muscle.

## METHODS

Five adult male patients from the peripheral vascular disease clinic were used as subjects. Each patient was kept at complete rest on a couch for 30 minutes in a warm room ( $22^{\circ}\text{C.} \pm 1^{\circ}$ ). When blood flow tests of the calf were made, the circulation of the foot was eliminated by applying a tourniquet distal to the plethysmograph. The water in the container was maintained at an indifferent temperature ( $34^{\circ}\text{C.} \pm 1^{\circ}$ ).

A plethysmograph of the Abramson type was used on the calf; the apparatus designed by Kunkel and Stead (5) was better suited for the foot.

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As in previous experiments, the terms 'skin circulation' and 'muscle circulation' have been applied to the inflow curves obtained from the foot in the one case and the calf in the other. In the former, skin and subcutaneous tissue predominates; in the latter, it is muscle.

The following procedures were carried out: 1) Blood flow to the calf and to the foot in the resting or basal state. This is designated as the 'resting' or 'minimal blood flow.' 2) Blood flow to the calf and to the foot after a 10-minute period of arterial occlusion. The marked increase in blood flow during the phase of reactive hyperemia is designated as the 'maximal blood flow.' 3) Blood flow to the calf and to the foot after the injection of adrenalin in varying doses.

The injection of adrenalin was made into the femoral artery below Poupart's ligament. Sharp, 22-gauge needles made it a painless procedure in most instances. If pain was experienced after the needle puncture the injection was delayed until the pain had abated. Although the dose of adrenalin varied from 0.001 gamma to 15 gamma, the total volume made up in normal salt solution was always 0.2 cc.

The response of the arbitrarily designated skin and muscle circulation to an injection of adrenalin into the femoral artery was evident after the 8 or 10 seconds it took to complete the injection and to apply the collecting pressure. Successive curves were recorded every 15 to 20 seconds during the period of change in blood flow. This seldom lasted more than 3 minutes.

Values for the resting blood flow and the maximal blood flow of the calf were relatively constant and reproducible in every experiment and the response to the same dose of adrenalin could be duplicated on the same day or successive days. This was of considerable importance because only 3 or 4 intra-arterial injections were made on any one occasion, and as many as 4 to 5 sessions (spread over a period of 2 weeks) were needed to complete the studies of both foot and calf.

The operative procedure in each patient was an extirpation of the second and third lumbar sympathetic ganglia. This produces an effect from the level of the knee down. The completeness of the operation was checked by the changes in skin temperature and color, by an increase in the electrical resistance of the skin on the operated side, and by histological study of the tissues removed.

In 4 patients, blood flow studies were done on the same extremity before and after sympathectomy. In the other, the non-sympathectomized limb was the control.

## RESULTS

In the first 3 cases, the peripheral circulation was clinically adequate, and the maximal blood flow indicated the presence of a good vascular bed in both skin and muscle. In the remaining 2, with advanced vascular disease, tests indicated a markedly limited vascular bed.

*Case 1. G. K.*, 60 years old. Both feet had been severely frost-bitten 10 years before. All of the peripheral pulses were bounding in type and the circulation in the extremities appeared clinically normal. The most striking sign was profuse sweating.

The left lumbar sympathetic chain was resected first. Histological sections of the ganglia removed were reported as being normal tissue. Immediately following the

operation there was a marked increase in warmth, dryness and brightness of color of the left leg.

*Skin circulation.* There was a threefold increase in the resting blood flow to the foot one week after sympathectomy—from 2.1 cc. to 6.2 cc.<sup>2</sup> The maximal blood flow remained constant at 17–18 cc. and indicated an excellent bed of collateral vessels. Before operation the minimal effective constrictor dose of adrenalin in this case was 0.01 gamma. This produced a decrease in blood flow from 2.1 cc. to 0.9 cc. Stronger concentrations resulted in even greater change: Thus with 2 gamma, there was such intense constriction that no measureable blood flow<sup>3</sup> could be obtained. Following operation, 0.01 gamma remained the minimal effective constrictor dose.

TABLE 1. BLOOD FLOW IN LEG AND RESPONSE TO ADRENALIN

CASE	TIME	SKIN CIRCULATION (FOOT)			MUSCLE CIRCULATION (CALF)			
		RBF <sup>3</sup>	MBF <sup>4</sup>	CD <sup>5</sup>	RBF	MBF	CD	DD <sup>6</sup>
1, G. K. <sup>1</sup>	Before S <sup>2</sup>	2.1	17	0.01	2.7	17	4	2.0
	8 Days post S	6.2	18	0.01	3.5	18	2	0.01
	22 Days post S	9.2		0.01	2.9		1	0.001
2, S. R. <sup>1</sup>	Before S	1.2	11	2.0	1.9	15.2	8	0.01
	33 Days post S	6.3	12	0.1	1.1	15.8	1	0.001
3, P. G.	Before S	3.4	9.8	0.1	2.1	21.6	4	1.0
	120 Days post S	6.3	13.8	0.1	2.1	20	1	0.01
4, H. G. <sup>1</sup>	Before S	1.0		1.0	0.8	3.5	8	0.01
	31 Days post S	3.0		1.0	0.8		2	0.002
5, J. McI. <sup>1</sup>	Before S				1.5	6.0	8	1.0
	30 Days post S				1.2	6.5	1	0.01

<sup>1</sup> Same foot used. <sup>2</sup> Sympathectomy. <sup>3</sup> Resting blood flow in cc/100 gm. tissue/min.

<sup>4</sup> Maximal blood flow in cc/100 gm. tissue/min. <sup>5</sup> Minimal effective constricting dose in gamma adrenalin. <sup>6</sup> Minimal effective dilating dose in gamma adrenalin.

Ten days post-sympathectomy it produced a decrease from 6.2 cc. to 2.6 cc. and 26 days post sympathectomy from 9.2 cc. to 4.8 cc.<sup>4</sup>

*Muscle circulation.* Prior to sympathectomy, 2 gamma adrenalin was a dilating dose and 4 gamma a constricting dose in the calf<sup>5</sup>. A blood flow of 2.7 cc. at rest was

<sup>2</sup> In cc/100-gm. tissue/min.

<sup>3</sup> This is not meant to indicate complete cessation of blood flow, merely the inability to record a markedly diminished flow with our recording system.

<sup>4</sup> Many of the intermediate doses as well as checks on the minimum effective doses are omitted from this report for brevity.

<sup>5</sup> Injection of 1–2 gamma of adrenalin in the normal forearm at the bend of the elbow produces vasoconstriction; doses of 0.05 to 0.0002 gamma produce vasodilatation. The reasons for the difference in dosage necessary to produce the same effects in the lower extremity appear to be 1) greater mass of tissue, 2) greater distance of calf from point of injection and 3) the presence of some degree of organic vessel disease. We did not feel that there were essential differences in the reactivity of vessels in the upper and the lower extremities.

elevated to 6.7 cc. by the former and diminished to an unrecordable level by the latter. Eight days after the operation 2 gamma proved to be a constricting dose and 0.01 gamma the minimal effective dilating dose. At this time the resting blood flow of 3.5 cc. was diminished to zero and raised to 9.6 cc. by the respective concentrations of adrenalin. Twenty-two days later, the resting flow of 2.9 cc. was elevated to 5.2 cc. by 0.001 gamma; 1 gamma produced intense vasoconstriction.

*Case 2.* This 61-year-old male, S. R., incurred frostbite of both hands and feet 35 years previously. All of the peripheral vessels were easily felt pulsating. Observations indicated the presence of Raynaud's syndrome, precipitated by the cold trauma of many years before.

Following a lumbar sympathectomy on the right there appeared the customary signs of vasoconstrictor release. Microscopic slides again showed only normal sympathetic ganglia.

*Skin circulation.* The resting blood flow of 1.2 cc. before operation rose to 6.3 cc. 33 days after sympathectomy. The maximal blood flow was 11-12 cc. indicating a fair collateral bed. The minimal effective constricting dose of adrenalin, intra-arterially injected, was 2 gamma before operation and 0.1 gamma after operation.

*Muscle circulation.* Resting blood flow in the calf before operation was 1.9 cc.; maximal flow was 15.2 cc. The minimal effective constrictor dose of adrenalin was 8 gamma before operation and 1 gamma 33 days later. Both produced intense vasoconstriction which resulted in non-measurable flow. The minimal effective dilator dose before operation was 0.01 gamma which raised the blood flow from 1.9 cc. to 4.5 cc. Thirty-three days after sympathectomy, the effective dilating dose was 0.001 gamma which raised the blood flow from 1.1 cc. to 3.2 cc.

*Case 3.* P. G., a 20-year-old male with thrombo-angiitis obliterans, developed an ulcer on the small toe of his left foot. None of the pedal pulses were felt on this side. A lumbar sympathectomy was performed for relief of the chronic ulcer. Our studies were done 4 months after the operation. The right lower extremity, with pulsatile vessels, was used as control.

*Skin circulation.* The resting blood flow was 3.4 cc. in the control foot and 6.3 in the sympathectomized foot. The maximal flows were 9.8 cc. and 7.6 cc. respectively. An intra-arterial injection of 0.01 gamma of adrenalin produced a decrease in blood flow to 1.5 cc. in the control. This was the minimal effective dose. On the sympathectomized foot, the same dose produced marked constriction and a non-measurable flow.

*Muscle circulation.* In the control calf, the resting blood flow was 2.1 cc. and the maximal flow 21.6 cc.; on the sympathectomized side the resting flow was 2.1 and the maximal flow 20 cc.

Intra-arterial injection of 1 gamma in the control limb raised the flow to 7 cc.; 4 gamma diminished the flow to 0.9 cc. and 2 gamma produced no change in flow. In the sympathectomized limb, 1 gamma reduced the flow to zero; 0.01 gamma elevated the flow to 4.2. A dose of 0.001 gamma was ineffective.

*Case 4.* H. G., age 40, had Buerger's disease. In 1946 after injury to his right ankle an ulcer developed and continued to spread. Both femoral pulsations were present but not the popliteal or pedal pulses. A right lumbar sympathectomy was performed.

The histological report indicated normal sympathetic ganglia. The right foot became warm and pink and the skin temperature of the toes rose to 87° F.

*Skin circulation.* The resting blood flow in the foot before sympathectomy was 1.0 cc. After operation, the flow was 3.0 cc. An exceedingly painful ulceration of the ankle which became even more painful with vascular occlusion hampered us in efforts to obtain the maximal blood flow. One gamma of adrenalin was the constricting dose and 0.01 gamma the ineffective dose, both before and after sympathetic section.

*Muscle circulation.* The resting blood flow in the right calf before operation was 0.8 cc.; after operation it was unchanged. A maximal flow was obtained in one test, and reached only 3.5 cc. This was a poor rise confirming the clinical evidence of a markedly impaired vascular reservoir. With 1 gamma before operation the blood flow in the calf rose to 1.6 cc. With 2, 4 and 8 gamma the flow increased to a maximum of 2.0 cc. Higher doses were not used, so that the minimal effective constrictor dose was not reached. In contrast, however, after sympathectomy, 2 gamma resulted in a non-measurable blood flow. The minimal effective dilating dose before operation was 0.01 gamma, after operation 0.002 gamma.

*Case 5. J. McI.,* age 51, had arteriosclerotic peripheral vascular disease with superimposed frostbite. A sympathectomy was done on the right side because of non-healing ulceration of the toes. This caused the foot to become warmer and drier and led to complete healing of the ulcers. Our tests were done on the same limb before and after sympathetic section.

*Skin circulation.* —Not attempted because of painful ulcerations of toes.

*Muscle circulation.* Resting flow values were about the same, 1.5 cc. and 1.2 cc., in both the control state and after sympathectomy. The maximal blood flow during reactive hyperemia reached 6 and 8.2 cc. in each instance. The minimal effective dilating dose before operation was 1 gamma. After operation this dose (in the same leg) resulted in a non-measurable blood flow. The minimal effective constricting dose before operation was 8 gamma. The minimal effective dilating dose post-sympathectomy was 0.01 gamma.

## RESULTS

The reported observations indicate the development of an increased sensitivity of blood vessels to adrenalin after the usual type of lumbar sympathectomy in man. This sensitivity may involve 1) constrictor mechanism of skin vessels, 2) constrictor mechanism of muscle vessels and 3) dilator mechanism of muscle vessels. Apparently vessels of the skin react to even minute doses of adrenalin by constriction so that we have been unable to elicit a dilator action as occurs in muscle.

An augmented responsiveness is clearly evident for the circulation of muscle in each of our subjects. In the skin, however, only in *case 2* is there a change of sufficient magnitude to warrant such a conclusion. In the remaining cases, a more complete but not impressive response results from the minimal effective constricting dose following sympathectomy. In *case 2*, Raynaud's phenomenon had been present before sympathectomy, with its implications of increased constrictor responses of skin blood vessels apart from those produced by operative intervention. This makes evaluation of increased constrictor responses after operation uncertain.

Sensitization of the muscle circulation to dilator doses of adrenalin appears much more marked than to constrictor doses. While the post-operative range of the latter

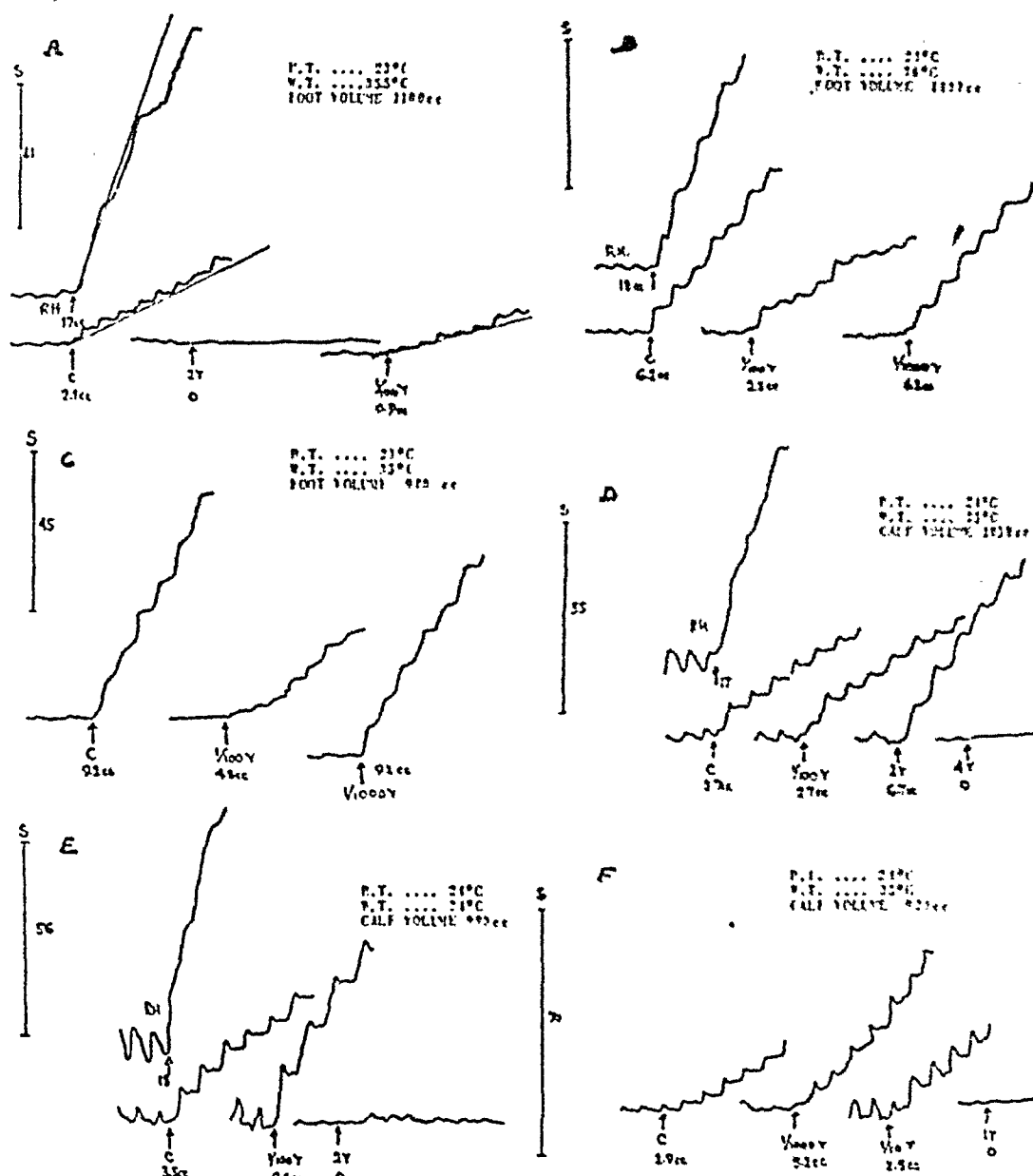


Fig. 1. BLOOD-FLOW CURVES, case 1, in foot (skin circulation) and calf (muscle circulation), before and after lumbar sympathectomy. A, foot, before sympathectomy; B, foot, 10 days post-operative; C, foot, 26 days post-operative; D, calf, before sympathectomy; E, calf, 5 days post-operative; F, calf, 22 days post-operative. Adrenalin injected into femoral artery in doses as indicated. S, standard; C, control or resting blood flow; R. H., flow during reactive hyperemia; R. T., room temperature; W. T., water temperature.

seems to be one-half to one-quarter of the pre-operative effective dose, the former may be extended to one-hundredth of its previous level.

Lumbar sympathectomy with the present technique (removal of L2 and L3) is

automatically preganglionic in type (6). Our results point conclusively to the development of a hyper-reactivity of at least the muscle circulation to adrenalin, just as in post-ganglionic section.

The response of skin vessels in man differs from those of muscle in their behavior to adrenalin after sympathectomy. Whether this is due to an anatomical difference in nerve supply (7), or to dissimilar effector organs can only be a matter of surmise.

The 'neutral zone' between constrictor and dilator doses of adrenalin for muscle circulation is preserved after sympathectomy. In the normal extremity it was postulated that adrenalin stimulated a dilator as well as a constrictor mechanism controlling the vessels (4). The former prevails only with a low concentration of the drug; the latter comes into play with stronger concentrations. Intermediate doses which were ineffective represented, in our opinion, the equilibration of both actions. This concept is confirmed by our observations after sympathectomy.

The hyper-reactivity of muscle blood vessels after sympathectomy is of clinical interest. Although vasodilatation is conspicuously absent in these vessels as a direct result of denervation, theoretically however, as a result of their increased sensitivity it is possible that vasodilatation might occur with physiological concentrations of humoral adrenalin. This deserves consideration as the cause of improvement in some patients with intermittent claudication who have had a sympathectomy. After denervation, constricting doses of adrenalin for muscle are well outside the physiological range and sensitization of skin vessels is too inconsistent to be of clinical significance.

#### SUMMARY

Blood flow in the sympathectomized human limb was studied by the plethysmographic method with varying doses of adrenalin injected into the femoral artery. Sensitization of muscle circulation is established and is greater for the dilator than for the constrictor mechanism. Physiological and clinical implications of these observations are briefly discussed.

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## HYDROLYSIS OF CHOLINE ESTERS IN THE PRESENCE OF ADRENALIN

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THE inhibition of cholinesterase activity by adrenalin was reported recently (1). The esterases used in these studies represented what were termed 'specific' and 'non-specific' cholinesterases. They were obtained, respectively, from the caudate nucleus and the parotid gland of the hog. The substrate used was acetylcholine.

It was considered desirable to study further the inhibitory effect of adrenalin using additional substrates and cholinesterase of other sources. This paper reports upon the findings of these studies.

### METHODS

The Warburg manometric technic was employed, following the procedure outlined in the previous publication (1). Two of the enzymes consisted of preparations of cholinesterase obtained from the caudate nucleus and the parotid gland of the hog. A third esterase, one also obtained from the parotid gland, differed from the one described above in that it was brought to dryness over calcium chloride rather than lyophilized in the final stage of preparation. All of these preparations were prepared in part similar to the technic of Mendel and Mundell (2) for the purification of a pseudo cholinesterase from dog pancreas.

In addition to these, the fresh frozen caudate nucleus of the dog was used for a further comparison of enzyme activity. The latter tissue was homogenized and diluted with Krebs-Henseleit (3) buffer solution. The activity of varying but known quantities of this tissue, approximating 5 mg. of wet weight, was followed in individual Warburg flasks. The tissue hydrolyzed acetylcholine

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and metacholine (acetyl-beta-methylcholine) readily. It also hydrolyzed benzoylcholine to a minor extent but was considered similar to the more purified hog caudate nucleus as representative of the 'specific' type of cholinesterase. The parotid preparations were of the 'non-specific' type. All of the enzyme preparations were dissolved or suspended in Krebs-Henseleit solution.

The substrates, consisting of acetylcholine, methacholine and benzoylcholine, were made up in buffer solutions. They were added in 0.5 ml. amounts (to make a final 0.003 molar concentration) to a side arm of their respective double sidearm flasks. Adrenalin hydrochloride, prepared from its base, was added in 0.5 ml. amounts to the other side arm.

Three concentrations of adrenalin were studied: 0.0015, 0.003 and 0.006 moles/liter. Immediately after placing the adrenalin in the side arms of the Warburg flasks, the flasks and the manometers were gassed with a mixture of 5 per cent  $\text{CO}_2$ -95%  $\text{N}_2$  to prevent its oxidation. After equilibration at 37.5°C. the contents of the side arms were tipped into the reaction chamber. The addition of adrenalin preceded that of the substrates. Controls and blanks, including those for non-enzymatic hydrolysis, were run simultaneously.

Gas evolution, resulting from the liberation of acetic acid and its action on bicarbonate, was measured in the conventional manner. All experiments were run in duplicate. The results were expressed in microliters of  $\text{CO}_2$  released per mg. of dry weight of tissue in 60 minutes of time.

## RESULTS

The results of these studies are presented in table 1. As was reported previously, the ability of cholinesterase to hydrolyze acetylcholine is inhibited by the presence of adrenalin. This is true regardless of the source or type of esterase. With increasing concentrations of adrenalin, the activity is increasingly depressed. Enzyme from the caudate nucleus of the hog and dog, acting upon methacholine, is inhibited in a similar manner. Inhibition also occurs when the parotid gland preparations act upon benzoylcholine in the presence of adrenalin.

The percentage inhibition of the 4 cholinesterase preparations is presented in table 2. The values are based upon the averages of the duplicate determinations of table 1. The inhibition of cholinesterase by adrenalin is greater when acting on methacholine than when it is acting on acetylcholine. This is demonstrable with both the lyophilized preparation of the hog's caudate nucleus and the homogenized preparation of the dog's caudate nucleus. Conversely, the inhibition of cholinesterase by adrenalin is less when the esterase is acting on benzoylcholine than when it is acting on acetylcholine. This is evident with both the lyophilized and the calcium chloride-dried preparations of the parotid gland. These relationships exist at all concentrations of the inhibitor.

This finding is more striking when it is appreciated that the degree of inhibition is not related to the rates at which the cholinesterases hydrolyze a given choline ester. A comparison of the lyophilized preparation of the hog's caudate nucleus (which represents a slow rate of catalysis) and the homogenized preparation of the dog's caudate nucleus (which represents a fast rate) bears this out. The degree of inhibition is similar whether normal catalysis is slow or fast.

The activity of two different types of esterases, the homogenized 'specific' esterase of the dog's caudate nucleus and the lyophilized 'non-specific' esterase of the hog's parotid gland, fortuitously, hydrolyzed acetylcholine at the same rate. It is, therefore, possible to compare directly the rates at which the one enzyme metabolizes methacholine and the other metabolizes benzoylcholine. It may be seen from table 1 that their activities in this respect are equivalent. In plotting the average percentage

TABLE 1. ACTIVITY OF VARIOUS CHOLINESTERASE PREPARATIONS IN THE PRESENCE OF ADRENALIN<sup>1</sup>

PREPARATION	SUBST. <sup>2</sup>	CONTROL	CONCENTRATION OF ADRENALIN		
			0.0015M	0.003M	0.006M
Caudate nucleus (Lyophil.; hog)	Ach	39.4	33.5	30.4	24.5
		36.5	33.3	28.4	23.4
	Mch	5.2	3.9	2.5	1.9
		5.0	3.1	1.9	0.7
Caudate nucleus (Homog.; dog)	Ach	206.5	200.9	145.7	126.3
		198.8	156.4	136.4	126.2
	Mch	60.4	46.9	40.8	32.5
		60.2	44.4	40.6	30.7
Parotid gland (Lyophil.; hog)	Ach	205.4	172.9	147.7	109.7
		201.4	171.7	147.0	106.0
	Bch	61.0	61.8	53.5	47.8
		60.8	58.7	52.6	45.8
Parotid gland (dried; hog)	Ach	217.6	194.3	172.7	140.1
		214.8	190.7	169.8	127.6
	Bch	74.5	65.1	64.4	58.1
		71.6	67.0	63.3	56.2

<sup>1</sup> Activity is expressed in microliters of CO<sub>2</sub> released in 60 min. of time per mg. dry wt. of preparation.

<sup>2</sup> Ach, Mch and Bch denote the substrates acetylcholine, methacholine and benzoylcholine in 0.003M concentration.

TABLE 2. PERCENTAGE INHIBITION OF CHOLINESTERASE BY ADRENALIN<sup>1</sup>

PREPARATION	CONCENTRATION OF ADRENALIN					
	0.0015M		0.003M		0.006M	
	Ach	Mch	Ach	Mch	Ach	Mch
Caudate nucleus Lyophil.; hog	12.1	31.0	22.7	56.5	36.9	74.5
Homog.; dog	11.8	24.3	30.4	32.5	37.7	47.6
Average	12.0	27.7	26.6	44.5	37.3	61.1
Parotid gland Lyophil.; hog	15.3	1.1	27.6	12.9	47.0	24.1
Dried; hog	10.9	9.6	20.8	12.6	38.1	21.8
Average	13.1	5.4	24.2	12.8	42.6	23.0

<sup>1</sup> Percentage inhibition is based upon averages of activities expressed in table 1.

of cholinesterase inhibition against the concentrations of inhibitor agent, as was done in figure 1, it is obvious that a marked difference in the two enzyme systems exists.

With methacholine as the substrate for the 'specific' type of esterase, the degree of adrenalin inhibition is high. With benzoylcholine as the substrate for the 'non-specific' esterase, the degree of inhibition is low.

#### DISCUSSION

The cholinesterases, in general, have been considered as a group of related and yet individual enzymes (4-6). They have been classified on the basis of the relation of their activity to the substrate concentration (5-8). The one type is characterized by maximum hydrolysis of acetylcholine at about  $3 \times 10^{-3}$  molar concentration. Inhibition occurs at concentrations above this. The other type is characterized by maximum hydrolysis at infinite substrate concentration. Unfortunately, the group numbers used by Augustinsson (6) are just the reverse of those used by Bodansky

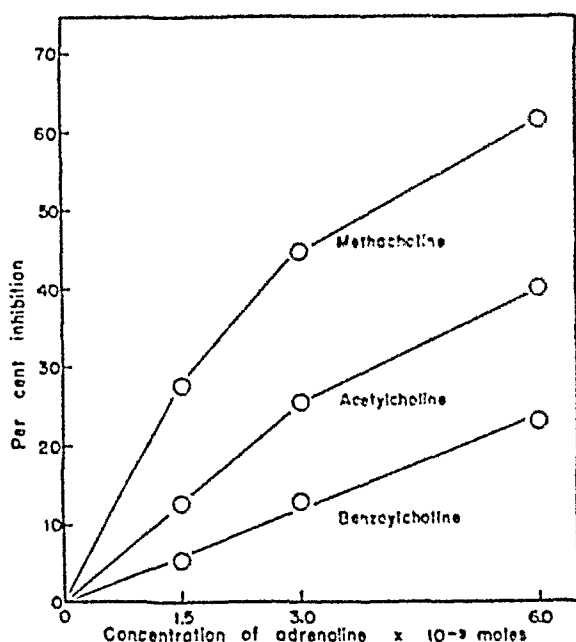


Fig. 1. INHIBITION OF CHOLINESTERASE by varying concentrations of adrenalin

(5). The enzymes of the caudate nucleus and the parotid gland used in this report correspond respectively to the above two groups. To avoid further confusion by numbering them, and yet to indicate the type of esterase to which they have been considered to belong in the past, the terminology of 'specific' and 'non-specific' cholinesterases has been retained in this paper. In view of the fact that both types of esterases are able to hydrolyze non-choline esters (5, 9) it may be expedient in the future to refer to these as *Groups A* and *B*, respectively.

The low order of activity of the lyophilized preparation of the caudate nucleus of the hog is of interest. This material lost over 50 per cent of its original activity when stored at  $4^{\circ}\text{C}$ . for a period of 2 months. It was also readily inactivated by heat. This is in marked contrast to the parotid preparations, which were only partially inactivated when heated at  $110^{\circ}\text{C}$ . for 24 hours. The 'specific' type of esterase is thus more readily inactivated than the 'non-specific' type. Studies on the thermolability and relative thermostability of the two esterases will be reported elsewhere.

It is of considerable interest that the two different esterases are inhibited by adrenalin to a similar degree when acting upon acetylcholine but to markedly different degrees when acting upon their respective substrates, methacholine and benzoylcholine. Since Augustinsson (6) has shown with dog's brain that the substrate concentration for optimum hydrolysis of acetylcholine and methacholine are very nearly the same, the difference in the degree of inhibition with different substrates suggests that the process involves factors other than competition for reactive groups. Preliminary studies indicate that the inhibition is non-competitive in nature.

#### SUMMARY

Adrenalin in concentrations varying from 0.0015 to 0.006 moles/liter inhibits the ability of cholinesterase of various sources to hydrolyze choline esters in 0.003 molar concentrations. With acetylcholine as the substrate, the degree of inhibition of 'specific' and 'non-specific' cholinesterases is of the same order of magnitude. With methacholine as the substrate for the 'specific' esterase, the degree of inhibition is comparatively high. With benzoylcholine as the substrate for the 'non-specific' esterase, the degree of inhibition is relatively low.

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# INACTIVATION OR REMOVAL OF INSULIN BY THE LIVER<sup>1</sup>

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IT WAS pointed out by Collens and Murlin (1) and Neuwirth, Co Tui and Wallace (2) that if one judged the effect of insulin by means of change in the blood sugar, then the intraportal injection of the hormone was less effective, unit for unit, than the injection into a peripheral systemic vein. These same authors, as well as Burger and Kramer (3), also found that a temporary initial rise in blood sugar is more frequently observed after intraportal insulin injections (single or repeated), than after injections via a systemic vein.

The realization that samples of insulin contain variable amounts of a hyperglycemic, glycogenolytic factor (H.F.) (4) might lead to the assumption that the intraportal injection of insulin was only apparently less effective, the expected hypoglycemia being masked by a simultaneous glycogenolytic action of the H.F. On the other hand, the intraportal route may be less effective because insulin itself may be partially inactivated or otherwise changed by its passage through the liver (5-7).

We tested these two possibilities by comparing the effectiveness of equal amounts of insulin injected continuously via the femoral vein and the splenic vein in normal and depancreatized animals. The participation of the hyperglycemic factor was tested by using two brands of insulin—one containing, and the other free of H.F.

## METHODS

All experiments were done on dogs under pentobarbital anesthesia. Depancreatized animals were used after a 72-hour period free of food and insulin. Comparisons between intrasplenic and intrafemoral injections were made on the same animal at 3-day intervals. The sequence of injection routes was varied and had no apparent effect on the results.

Preliminary experiments were done with the dose of insulin varying from 1/5 to 1/50 U/kg/hr., given as a constant injection in 52 cc. of saline per hour for 2 hours. The experiments reported were done with 0.1 U/kg/hr. since this dose gave definite drops in blood sugar via both injection routes. Arterial blood samples were analyzed in duplicate for glucose by the Somogyi modification (8, 9) of the Shaffer-Hartmann Technique.

## RESULTS

Our preliminary experiments revealed that there was a gradation of effect of the injected insulin. The minimum effective dose was found to lie between 1/50 and 1/25 U/kg/hr.

Figure 1 presents the results in normal dogs injected with 0.1 U/kg/hr. of Lilly insulin, known to contain H.F. (4). It can be seen that there is a small but definite

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<sup>1</sup> A preliminary report of this work appeared in *Federation Proc.* 8: 52, 1949.

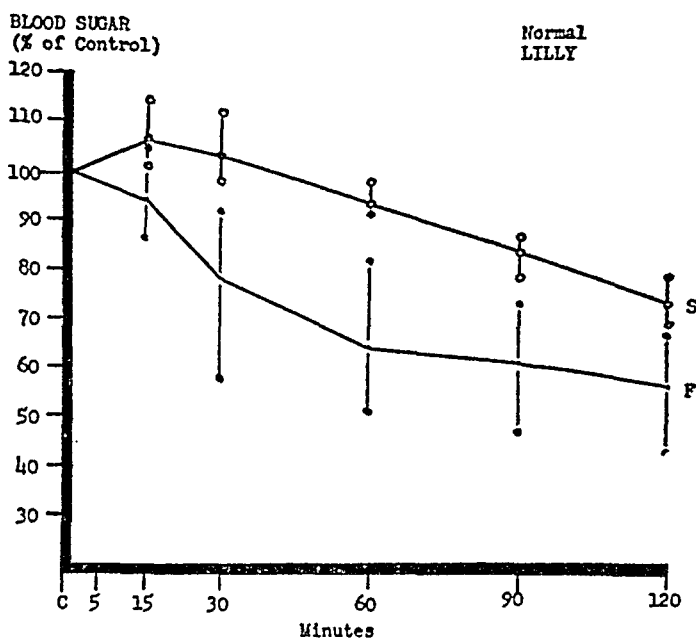
<sup>2</sup> The department is supported in part by the Michael Reese Research Foundation.

initial rise during the first 15 minutes of the splenic infusion. The rate of fall between 15 and 60 minutes was greater when the femoral route was used. The net result is that the femoral route gives a significantly greater blood sugar depressing effect than does the splenic route. Despite absolute variations, this was the case in each animal used.

Essentially the same results were obtained when these experiments were done on 2 totally depancreatized dogs (fig. 2). The fasting blood sugar levels of these animals varied from 272 to 519 mg.%, but in order to compare the results with the normal group, the curves are plotted as percentage change from the pre-injection value.

Figure 3 presents similar data on 2 normal dogs given the same amount of insulin (Novo) not containing any appreciable amount of H.F. It can be seen that the blood sugar depressing effect of this insulin is significantly greater than that of the previous

Fig. 1. EFFECT OF 0.1 U/KG/HR. of Lilly insulin in normal dogs. *Upper curve:* splenic vein injection (3 exper.). *Lower curve:* femoral vein injection (7 exper.). Range of values shown for each curve.



brand, by both routes of administration. However, even in the absence of demonstrable H.F., the difference in the effectiveness of the femoral vs. the splenic routes is preserved.

Table 1 expresses the insulin effect in terms of the area of the curve. The ratio of effect of the splenic route to the femoral route is seen to be 1:3.85, 1:1.82 and 1:1.83 for the three sets of experiments.

#### DISCUSSION

It is apparent from the data presented that the lessened effectiveness of a given dose of insulin when injected intraportally as compared to an intrafemoral injection cannot be completely ascribed to the masking action of a glycogenolytic effect of an admixed H.F. Comparison of figures 1 and 3 shows that there is a greater fall in each curve when Novo insulin is used. The data in table 1 show that the femoral route is 3.85 times as effective for Lilly insulin in contrast to 1.83 times for Novo. This

may be explained by the presence of the H.F. which causes the liver glycogen to fall and the blood sugar to rise, thus resulting in a small drop in the blood sugar when insulin is given to normal animals via the splenic vein.

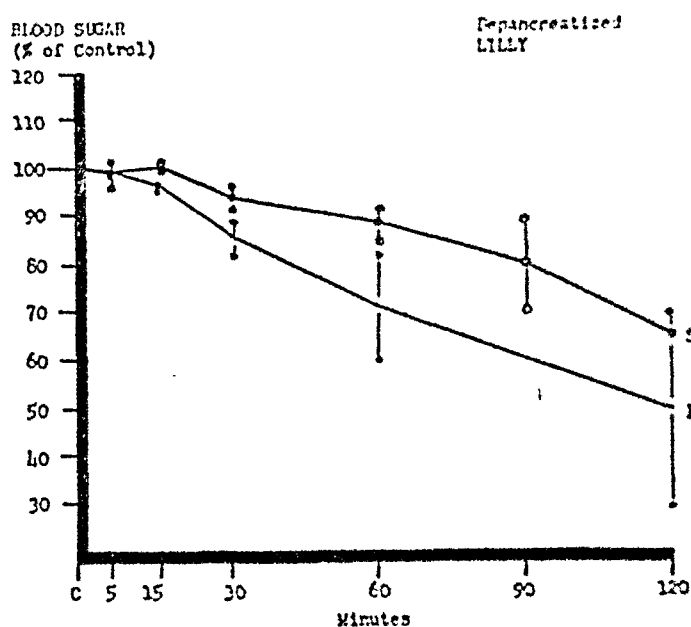


Fig. 2. EFFECT OF 0.1 U/KG/HR. of Lilly insulin in 2 totally de-pancreatized dogs. *Upper curve*: splenic vein injection. *Lower curve*: femoral vein injection.

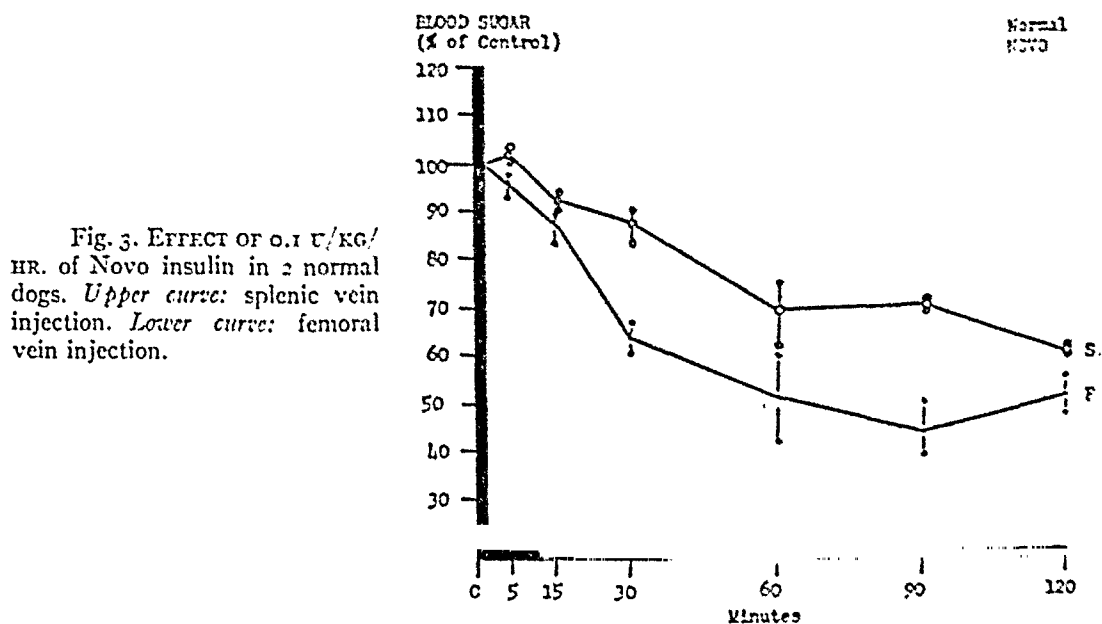


Fig. 3. EFFECT OF 0.1 U/KG/HR. of Novo insulin in 2 normal dogs. *Upper curve*: splenic vein injection. *Lower curve*: femoral vein injection.

The liver apparently either retains or inactivates a portion of the insulin which comes to it through the portal vein. This is consistent with the findings of Broh-Kahn and Mirsky of the presence of an enzymatic system ('insulinase') in the liver capable of destroying the insulin molecule (5-7). They reported that liver contains the greatest 'insulinase' activity; kidney and muscle less. The enzyme system was found in the liver of the rat, rabbit, steer, chicken and man.

Insulinase will explain the differences observed in splenic *vs.* femoral injection. Despite the effect of the H.F. the inactivation or removal of insulin by the liver is seen even when Novo insulin was used. The area difference between splenic and femoral (table 1) is 22.3 and 18.8 for Lilly and Novo insulin, respectively. The difference for the depancreatized animals is 12.1.

Broh-Kahn and Mirsky (7) showed that fasting causes a reduction in insulinase activity of the liver. This explains the results obtained in our depancreatized dogs which were without food for 72 hours. Deprivation of food for this period of time is sufficient to cause a decreased liver insulinase activity so that less insulin would be inactivated. This is seen in the 7.8 and 14.7 units of area for the normal and depancreatized splenic injections, respectively. These two groups did not fall as low as the Novo group (22.6) because of the presence of the H.F. The decreased insulinase activity is also demonstrated in the small (12.1) difference in area between splenic and femoral injections in the depancreatized group.

Our screening experiments showed that 1/25 to 1/50 U/kg/hr. had very little effect in lowering the blood sugar. This compares favorably with Slater *et al.* (10)

TABLE 1

CONDITION OF ANIMALS	TYPE OF INSULIN	AREA <sup>1</sup>		DIFFERENCE	RATIO OF EFFECT Splenic:Femoral
		Splenic	Femoral		
Normal	Lilly	7.8	30.1	22.3	1:3.85
Depancreatized	Lilly	14.7	26.8	12.1	1:1.82
Normal	Novo	22.6	41.4	18.8	1:1.83

<sup>1</sup> By 'area' is meant the area enclosed by the blood sugar curve and the horizontal line passing through 100 in figs. 1, 2, and 3, expressed in arbitrary units.

who found that a single intravenous dose of 1/40 U/kg. in normal dogs caused either no change or only a slight temporary drop in blood sugar.

It may be that the dog liver insulinase is capable of inactivating about 1/25 to 1/50 U/kg. body wt/hr. of insulin.

#### SUMMARY AND CONCLUSIONS

Under normal circumstances insulin, after leaving the pancreas, reaches the liver before it goes into the general circulation. Our evidence shows that the liver removes or inactivates insulin as it passes through the liver. This is probably via the 'insulinase' system described by Mirsky and Broh-Kahn. Liver inactivation of insulin is demonstrated in normal and depancreatized animals. This action is found despite the presence of the hyperglycemic factor in certain insulin preparations. It is conjectured that the liver of the dog can inactivate about 1/25 to 1/50 U/kg. body wt/hr. of insulin.

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# SEASONAL CHANGES IN THE THYROID GLAND AND EFFECTS OF THYROIDECTOMY IN THE MALLARD, IN RELATION TO MOULT

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A NUMBER of observers have shown that the administration of thyroid or thyrotropic preparations can bring on a moult outside the normal moulting season in domestic fowl (1-3). Others have shown that thyroidectomy can postpone the moult in chicks (4). Thyroidectomy has also been shown to postpone the moult or to abolish it altogether in a number of other species of birds (5, 6).

These findings have naturally led to the supposition that in birds generally increased thyroid activity is responsible for the shedding of the worn plumage and its replacement by new feathers, which constitute the moult. However, it does not appear that this supposition has yet been proved conclusively in the case of any one species. The findings in anserine birds (swans, ducks and geese) in particular are not in agreement with the view that thyroid activity is responsible for the moult in this group. The observers who have administered thyroid preparations to ducks agree that a moult is not precipitated in these birds by doses which produce this effect in fowls (7-9). Further, Chu (10), referring to unpublished work and therefore without giving further details, reports that adult mallards after complete thyroidectomy still moulted into the next plumage. On the other hand, Woitkewitsch (6) found that thyroidectomy in young ducklings prevented the moult from down into the first true plumage if the operation was performed well before the expected onset of the moult. It should also be pointed out that there is fairly convincing evidence that the onset of the moult in the mallard is controlled by the gonads, since a number of observers have found an indefinite postponement of the moult after castration (see review in 10); Chu was able to bring on a moult by administration of pituitary gonadotrophins.

The work here reported is an attempt to clarify the rôle of the thyroid in the moulting process of the mallard. The mallard has two annual moults. The first takes place in June to July in males; in this the entire plumage is renewed in the change from the mating- or winter- to the eclipse-plumage of summer. In females this moult is delayed to July to August (until the young are partly fledged). A second less extensive moult occurs in the fall; in males August to November, in females October to March. This moult affects the body plumage and only a few of the larger feathers and may be spread over a long period. Moulting periods as given apply to English mallards (16) such as were used for this work. A study of seasonal changes in the thyroid in relation to these plumage changes seemed of particular interest, since on the supposition of a thyroid control of the moult a period of increased thyroid activity would be expected to precede the two moults. In view of the thyroidectomy experiments of Chu and Woitkewitsch, it appeared desirable to investigate the effect of this operation on the moult in somewhat older birds than those used by the second observer.

TABLE I. CONDITION OF THYROID GLAND IN RELATION TO PLUMAGE

DATE	THYROID WT. MG. <sup>1</sup>	THYROID STATE	PLUMAGE TYPE
<i>A. Males</i>			
1/11	110	Intermed.	Mating
1/15	87	Active	Mating
2/15	128	Storage	Mating
3/15 <sup>2</sup>	115	Storage	Mating
4/11	45	Active	Mating
5/15	187	Active	Mating
5/24	135	Active	Mating
6/7	114	Storage	Early moult
6/19	75	Storage	Moult
6/21	170	Storage	Moult
7/8	90		Moult
7/16	135	Active	Moult
7/19	150	Storage	Moult + eclipse
7/31	81	Storage	Eclipse
8/2	220	Storage	Eclipse
8/10	100	Storage	Eclipse + 2nd moult
8/13	107	Intermed.	Eclipse + 2nd moult
9/17 <sup>3</sup>	89	Active	Nearly full mating
10/15	135	Active	Mating
10/23	95	Active	Mating
11/15	108	Active	Mating
11/20	70	Storage	Mating
11/20	135	Active	Mating
11/23	135	Intermed.	Mating
11/28	80	Active	Mating
11/29	70	Active	Mating
12/11	110	Intermed.	Mating
12/12	88	Active	Mating
<i>B. Females</i>			
1/18	59	Intermed.	Mating + nest down growing
2/19	140	Intermed.	Mating + nest down present
2/22	110	Active	Mating + nest down growing
3/22	113	Storage	Mating
4/24	107	Storage	Mating
5/19	160		Mating
5/24	122		Mating
5/25	126	Intermed.	Mating
6/7	133	Active	Mating
6/21	40	Active	Mating
6/26	72	Active	Mating
7/8	120	Storage	Buds of Eclipse
7/19	107	Storage	Buds of Eclipse
8/2	155	Intermed.	Eclipse
8/10	90	Intermed.	Eclipse
10/30	100	Active	Mating
11/15	—	Active	Mating
12/18	95	Active	Mating

<sup>1</sup> Both lobes.    <sup>2</sup> This bird had a dermatitis with loss of feathers on the neck.    <sup>3</sup> This bird showed signs of a chronic paracercitis.

## SEASONAL CHANGES IN THE THYROID GLAND

*Material and Methods.* Twenty-eight adult male and 16 adult female mallards (*Anas platyrhynchos* L) were shot at regular intervals in one of the London Royal Parks. Freshly dissected thyroid glands were weighed on a torsion balance, fixed in Bouin and stained with Heidenhain's hematoxylin and eosin. Küchler (11), using

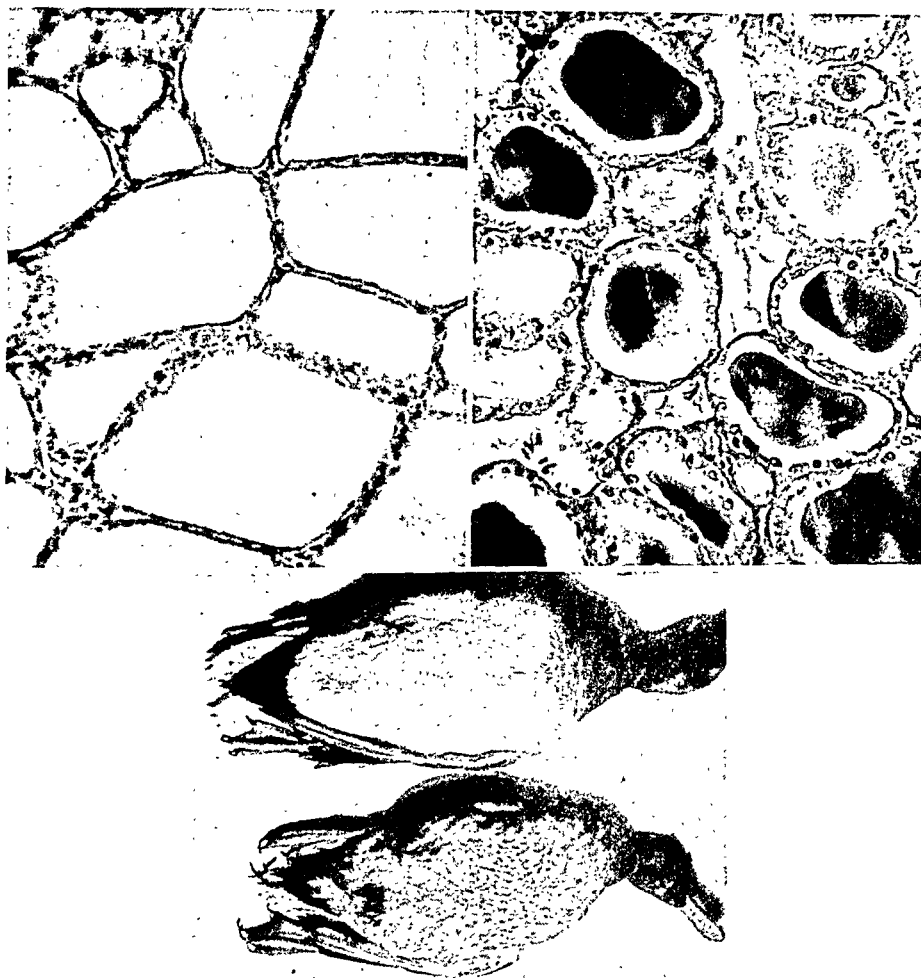


Fig. 1. *Upper left:* Photomicrograph of adult female mallard's thyroid in 'resting,' relatively inactive condition  $\times 210$  (Aug. 2, 1944). *Upper right:* Adult female mallard's thyroid in 'active' condition  $\times 210$  (Feb. 19, 1946). *Lower:* Ventral views of control male, top, in full breeding plumage; thyroidectomised male, bottom, in breeding plumage on head, neck, upper breast and tail but retained juvenile plumage on abdomen. 'Loose' feathers characteristic of hypothyroidism. Photographed 6 months after thyroidectomy.

this method on avian material, claims that intra-cellular droplets of colloid are shown as colorless vacuoles. In the mallard it was not possible to demonstrate this material by this method nor by that of Dvoskin (12) in glands which by other criteria were highly active. Data on body weight, weight and histology of the gonads, adrenals and thymus of the birds used in the present series have already been reported elsewhere (13, 14).

*Results (Table 1 and figs. 2 and 3).* The macroscopic anatomy of the mallard's

thyroid conforms to the usual avian pattern of two entirely discrete lobes, one on each side, lying on the carotid artery. Three deviations from the normal arrangement were encountered, all in females. In 2 birds the lobes were connected at their caudal extremities by a thin strand of thyroid tissue running across the trachea. In another female, outside the present series, there was no thyroid on the left, the right lobe being about twice the normal in weight, so that the total amount of thyroid tissue was still about average.

*Thyroid Weight.* On the whole, glands which were judged active, in histological appearance, were below average in weight; while 'storage' glands were heavy. A complete correlation between weight and activity probably does not appear since a gland will show the picture of a 'storage' phase before colloid storage has caused a marked weight increase. Similarly, early in a secretory phase actual weight loss from

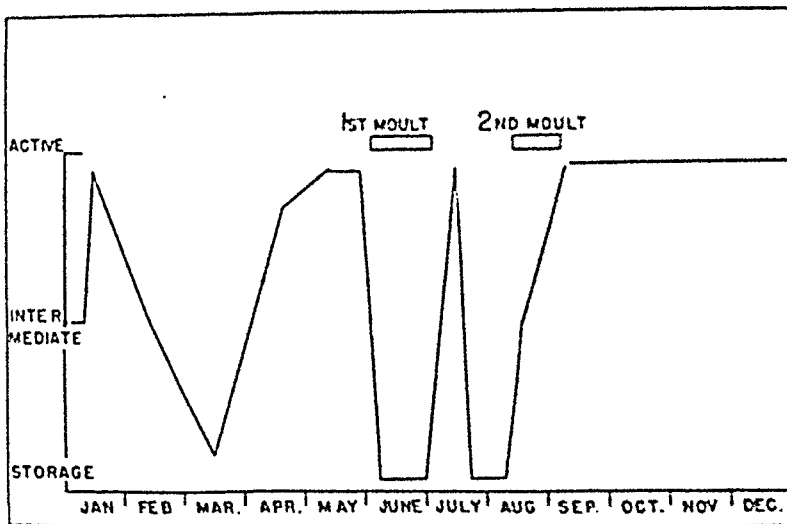


Fig. 2. SEASONAL ACTIVITY of thyroid of adult male mallards (based on histological findings) related to moults.

colloid depletion may be expected to lag behind the appearance of histological activity. Gland weights alone therefore give only a poor indication of the seasonal cycle of activity. The average weight of the thyroid tissue of 28 males was 108.37 mg. and 88.9 mg/kg. body weight. For females absolute average weight was 104.8 mg. and 100.8 mg/kg. body weight. It is doubtful whether this difference in thyroid weight per unit body weight is significant, since owing to unequal numbers and unequal seasonal distribution of the respective data for the two sexes, the figures may be biased.

*Histological Appearances.* A number of serial sections through the center of each of the two lobes were examined for every bird. For each lobe 10 measurements of vesicle diameter and 'vesicle wall thickness' (depth of tissue between two adjacent vesicles = twice epithelial height + intervening connective tissue  $\pm$  capillary, a ready index of epithelial cell height) were made. Vascularity was assessed by the number of non-capillary vessels in 10 high power fields per lobe. Although the histological technique used was unsuitable for cytological detail, two extreme phases of

thyroid activity could easily be recognized on this basis. These may be briefly characterized as follows, quantitative data being based on the above measurements for both thyroids of 10 birds, in each case both sexes being represented. 1) A resting or 'storage' gland with large vesicles average diameter  $79.5 \mu$  with low epithelium and flattened nuclei, average wall thickness  $6 \mu$  and poor vascularity, an average of 8 non-capillary vessels per 10 high power fields. This presumably represents a phase of colloid accumulation but with reduced colloid production and greatly reduced hormone discharge into the circulation. 2) The other extreme labelled 'active' was a phase of maximal secretory activity with increased hormone discharge. Vesicles were small, average diameter  $47 \mu$ , epithelium high, average wall thickness  $8.5 \mu$  with rounded, paler nuclei, a well-developed intervesicular capillary network and an apparent increase in larger vessels, average 23 in 10 high power fields. Such glands

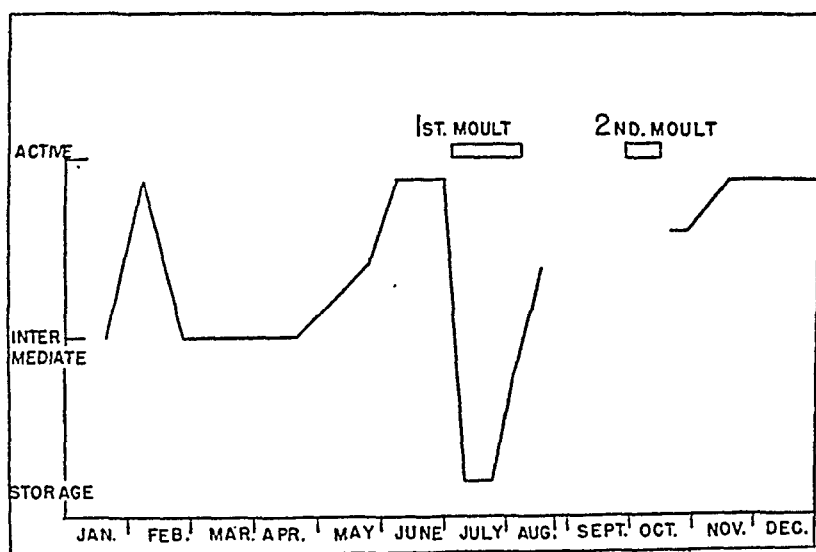


Fig. 3. SEASONAL ACTIVITY of thyroid of adult female mallards (based on histological findings) related to moults.

frequently showed non-staining, approximately circular areas in the peripheral colloid. These also appear in Zenker fixed glands stained with azocarmine and Heidenhain's iron-haematoxylin. Since they were always found in 'active' glands, the suggestion of others that they represent a transport phase of the colloid during secretion into the vesicle or during its resorption therefrom appears plausible though their precise nature cannot be regarded as established.

Table 1 summarizes the seasonal phases of gland activity as assessed by these histological criteria in relation to gland weights and plumage; while figures 2 and 3 represent the activity cycle in relation to the moults in graphic form.

Two clearly separated phases of thyroid activity are shown. First, during the colder months, October to January, there is a period of activity in both sexes. A similar increase of thyroid activity during the cooler months has been reported in the house sparrow by Miller (15), who has also induced this response experimentally by exposure to low external temperatures. Undoubtedly this response plays a part in temperature regulation during winter in birds.

Second, a phase of activity is found in the summer which appears to be related to the first moult. In the male, this phase extends from mid-April to late May, preceding the onset of the moult by about one month (fig. 2). In the female, it occurs later, namely in June, but again its onset precedes the moult by about one month (fig. 3). The fact that the summer phase of thyroid activity shows a different seasonal incidence in the two sexes is strong evidence that this activity is in fact correlated with the moult. If any phase of thyroid activity precedes the less severe second moult, it must be of much shorter duration than that preceding the major moult. The active condition of a male thyroid of July 16 may represent this phase. The fact that the phase of thyroid activity precedes rather than synchronizes with the moult is explicable, since an interval between the time of increased release of thyroid hormone and its effect on the rest of the body is to be expected. This expectation is confirmed by Woitkewitsch (6), who showed that in the starling, in which thyroidectomy abolishes the moult, the operation is effective only if it precedes the onset of the moult by a considerable period.

TABLE 2. EFFECT OF THYROIDECTOMY OF 2-MONTH OLD MALLARDS ON BODY WEIGHT AND MOULT  
(OPERATION JULY 15-18, 1947)

<i>Males</i>	
Operated: 750, 900 gm. <sup>1</sup>	Controls: 1050, 1000 1100, 950 gm.
Average: 825 gm. <sup>1</sup>	Average: 1025 gm.
<i>Females</i>	
Operated: 950 gm. <sup>1</sup>	Controls: 1000, 1025, 1000, 1050, 1000, 1150, 950 gm.
	Average: 1035 gm.

<sup>1</sup> Body weight when killed, 7 months old (Jan. 6, 1948).

#### RESULTS OF THYROIDECTOMY

Since Woitkewitsch (6) showed that thyroidectomy performed in ducklings when 5 to 10 days old prevented the moult completely, while on the other hand Chu (10) found that in adult mallards thyroidectomy did not prevent onset of the moult into the eclipse plumage, it was of interest to investigate the effects of thyroidectomy at an intermediate age. A number of mallards were, therefore, thyroidectomized when two months old, July 15 to 18, to observe the effect on the moult from the first juvenile into the first mating plumage due about mid-September. Three birds, 2 males and 1 female, made a complete recovery and survived until January 6, when they were killed. Post-mortem examination of the thyroid region (macroscopic and microscopic) showed that the operation had been complete and no thyroid regeneration had taken place. Table 2 summarizes the effects of the operation on body weight. This shows that the operation delayed growth as indicated by decreased body weight compared to the controls. The effect of thyroidectomy on the moult and formation of the new plumage is shown by the plumage conditions as recorded on November 25 (17 weeks after thyroidectomy of the experimental birds).

*Males.* Four controls in full breeding plumage, 1 thyroidectomized bird in full breeding plumage, the other showed retention of the juvenile plumage on the abdomen; otherwise full breeding plumage.

*Females.* Seven controls, all in full breeding plumage, thyroidectomized bird also in full breeding plumage.

The operated birds showed the usual structural feather defects of thyroidectomy. The date of first appearance of the new plumage was variable in the controls of both sexes and no definite lag in appearance of new feathers in the thyroidectomized birds was evident at any time.

Failure of the moult in part of the ventral surface as noted in one of the operated males occurs occasionally in wild mallards wintering in northern latitudes (personal communication of Prof. W. Rowan) and its occurrence in the present instance is, therefore, not necessarily attributable to the thyroidectomy. Hence, it must be concluded that thyroidectomy did not prevent or delay the onset of the moult.

#### DISCUSSION

The result of 3 thyroidectomy experiments may, by themselves, be regarded as inconclusive, but they are supported by the similar findings of Chu (10). The prevention or postponement of the moult in ducklings operated when under one month old, reported by Woitkewitsch (6), can readily be explained as part of the general retardation of development induced by thyroidectomy, rather than as a specific effect of the operation on the moulting process. The general conclusion that mallards can moult in the absence of the thyroid gland therefore remains unaffected.

Recent observations by Sulman and Perek (17, 18) show that the basal metabolism of hens is raised considerably during the moult. Nevertheless, when this was prevented by administration of thiouracil, the moult was neither postponed nor prevented. The position in the mallard appears to be essentially similar; increased thyroid activity occurs somewhat prior to the moult; by this, the metabolic rate is no doubt raised, presumably in connection with the formation of new feathers. This phenomenon is, however, merely associated with and not a cause of the moult, which still occurs after thyroidectomy. As indicated by the experiments of Chu referred to above, there is, however, a causal relationship between androgenic activity of the testis and the moult in this species.

#### SUMMARY

Histological observations on 24 adult male and 16 adult female mallards indicate two seasonal phases of increased thyroid activity: *a*) one affecting both sexes from October to January; *b*) one preceding the first, more extensive moult by about one month, occurring in males from April to late May and in females, which moult about one month later, in June. A second, much more transient period of increased thyroid activity may precede the less extensive second moult.

In three birds operated two months before the expected moult from juvenile to first breeding plumage which survived a 6-month period of observation after complete thyroidectomy, the moult was not prevented or postponed, though one bird failed to moult its abdominal feathers.

Three females showed a deviation from the normal macroscopic thyroid pattern, the two lobes being united caudally across the midline of the neck in 2 birds; in another the left thyroid was absent but the right hypertrophied. Mean weights of thyroid tissue were 88.9 mg/kg. body wt. in males and 100.8 mg/kg. body wt. in females.

Thanks are due to the medical research committee of Guy's Hospital Medical School (where the material was prepared) for financial support; to Dr. J. Beattie of the Royal College of Surgeons (England) for facilities to keep ducks at the Buckstone Brown Experimental Farm, and to Sir Arthur Keith and Mr. F. Watson for observations on these birds in the author's absence.

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# FLUCTUATIONS OF SERUM CHOLINE IN WOMEN

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SEVERAL lines of evidence suggest that variations in the choline content of serum occur throughout the menstrual cycle. Thus estrogens, which exhibit two maximal levels during the cycle, are known to affect the cholinesterase activity of blood (1-4) and to exert a cholinergic action upon certain tissues (5-8). This subject was recently reviewed comprehensively (9).

## PROCEDURES

In order to investigate the above relationship the serum choline content of 36 women was studied. As noted in table 1, there were two categories of subjects, designated *A* and *K* respectively. *Group K* comprised 15 subjects who were patients in the Gynecologic Department of the Municipal Hospital in Copenhagen. The patients selected for study presented insignificant gynecological anamneses and, practically speaking, may be regarded as normal healthy individuals. On each of these subjects a duplicate determination for serum choline content was made only once. *Group A* comprised 21 female medical students with no gynecologic complaints. On most of these subjects several duplicate determinations were made at various times (see table 1, column 3).

In all, 93 duplicate determinations were made. In order to insure uniformity all blood samples were drawn in the morning. In most instances the individuals were fasting when blood samples were drawn. A few by mistake had their breakfast beforehand but this did not seem to have any effect on the choline level of the blood.

Determinations of the choline content in serum were made by acetylation of the serum, whereby the choline was converted to acetylcholine. The method of Abdon and Ljungdahl-Østberg (10), slightly modified, was employed for the acetylation of serum. Blood samples were drawn by venous puncture. The amount of acetylcholine formed was determined by comparing the effect of the acetylcholine obtained by acetylation with the effect of a solution of acetylcholine of known potency on isolated guinea pig intestines (9). The mean of the standard deviation of the individual determinations in duplicate is 9.4 per cent, in single, 12 per cent (9).

## RESULTS

*Serum Choline.* It is readily apparent from table 1 that there was a wide spread of experimental results within the individual days of the cycle and from day to day. It was necessary to investigate whether or not these variations could be attributed, entirely or in part, to variations in the choline content of the diet.

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TABLE I

SUBJECT	DATE	SERUM CHOL- ESTERASE, MG. %	DAY OF CYCLE	SUBJECT	DATE	SERUM CHOL- ESTERASE, MG. %	DAY OF CYCLE
<i>Women Students</i>							
A 34	5/ 9/47	0.433	21	A 42	10/ 1/47	0.296	6
A 35	5/28/47	0.368	7		10/ 8/47	0.232	12
	4/28/47	0.242	5		10/23/47	0.728	27
A 37	4/28/47	0.185	26		10/29/47	0.555	5
A 38	5/ 9/47	0.467	4		2/ 3/48	1.210	15
	5/23/47	0.670	18		3/ 2/48	1.370	14
					3/10/48	2.145	22
A 40	5/23/47	0.400	1	A 48	10/ 6/47	0.204	18
	9/25/47	0.425	13		10/13/47	0.304	24
	10/ 2/47	0.462	20		11/ 3/47	0.420	15
	11/17/47	0.304	1	A 53	11/ 3/47	0.442	4
	11/24/47	0.433	7		11/10/47	0.104	11
	12/ 8/47	0.433	21		11/17/47	0.620	18
					11/26/47	0.383	27
A 41	9/24/47	0.308	6	A 48	11/ 6/47	0.357	22
	10/ 8/47	0.160	20		11/20/47	0.672	8
	10/15/47	0.288	25		11/27/47	0.388	15
	10/22/47	0.447	4	A 50	11/ 6/47	0.860	18
	10/30/47	0.500	12		11/27/47	0.620	12
A 43	9/24/47	0.395	12		12/ 3/47	0.372	17
	10/ 1/47	0.199	19		4/12/48	0.085	10
	10/ 8/47	0.170	4	A 51	11/ 5/47	0.343	21
	10/15/47	0.438	11		11/19/47	0.440	4
	10/22/47	0.402	18		12/ 2/47	0.565	17
	2/ 2/48	1.375	1		4/29/48	0.605	20
A 44	9/25/47	0.317	25	A 52	11/ 5/47	0.240	6
	10/ 2/47	0.370	26		11/10/47	0.603	20
	10/ 9/47	0.398	5		12/ 1/47	0.573	6
	10/16/47	0.408	12		12/10/47	0.695	15
	10/22/47	0.543	18	A 54	11/ 5/47	0.437	1
A 45	9/25/47	0.400	5		11/19/47	0.570	14
	10/ 9/47	0.342	19		12/ 3/47	0.380	28
	10/16/47	0.328	25		3/ 2/47	0.847	21
	10/29/47	0.414	10	A 55	11/20/47	0.257	5
A 46	9/29/47	0.317	2		11/27/47	0.858	12
	10/ 6/47	0.317	2		12/ 1/47	0.383	16
	10/27/47	0.582	9	A 57	12/10/47	1.036	13
	10/29/47	0.550	3		4/29/47	0.595	21
A 47	9/29/47	0.762	7				
	10/ 6/47	0.390	15				
	10/13/47	0.433	21				
	10/29/47	0.580	4				
<i>Clinic Patients</i>							
K 0	5/21/47	0.422	10	K 26	7/ 8/47	0.169	1
K 13	6/13/47	0.265	17	K 27	7/ 8/47	0.332	6
K 14	6/13/47	0.412	10	K 30	7/10/47	0.253	15
K 15	6/17/47	0.233	7	K 31	7/10/47	0.054	28
K 20	6/24/47	0.278	27	K 32	7/15/47	0.381	15
K 21	6/24/47	0.340	19	K 34	7/15/47	0.305	9
K 22	6/24/47	0.272	26	K 37	7/17/47	0.382	24
K 23	7/ 2/47	0.418	28				

*Diet and Serum Choline.* Experimenting with animals, Luecke and Pearson (11) found that a dosage of 400 mg. of choline chloride administered daily for 6 days did not raise the choline content of either the liver, kidney or plasma of sheep. Borglin (12) found no indication that the level of choline in human blood changes with the diet. Following the administration of pure choline in very high dosage he observed a sharp rise in choline content followed by a comparatively rapid decline to original levels. The chief purpose of this phase of our study was to ascertain whether or not a diet high in choline or choline-producing substances ingested on the day prior to the drawing of blood samples would influence the serum choline content in the morning of the following day.

For 5 consecutive days analyses of the serum choline content of one patient in the Municipal Hospital in Copenhagen were made. During the first 2 days the patient was given a normal hospital diet. During the following 2 days the patient received 5 gm. of L-methionine, 6 eggs and 130 gm. of protein daily. The results are given in table 2. The patient was a woman (K44, table 2) aged 17. She was admitted to the hospital on January 3, 1948, and was under observation for rheumatic fever. At the time when blood samples were drawn she was non-feverish and without complaints.

It appears from table 2 that the administration of a diet high in choline does not raise the serum choline content of a blood sample drawn the following morning. It is true that relatively great

TABLE 2

DATE	DIET	SODIUM	CHOLINE, MG. PER CENT
1/18/48	8 A.M.	fasting	1.42
1/18/48	7 P.M.	normal	1.40
1/19/48	8 A.M.	fasting	1.24
1/19/48	5 P.M.	normal	1.27
1/20/48	8 A.M.	fasting	0.90
1/20/48	6 P.M.	spec. diet	2.13
1/21/48	8 A.M.	fasting	0.81
1/21/48	4 P.M.	spec. diet	0.98
1/22/48	8 A.M.	fasting	1.70

variations are observed, but these variations do not seem to be attributable to the diet. The patient began to menstruate on January 19, 1948. It is possible that the variations in serum choline correspond to some change associated with the menstrual cycle. It is difficult, however, to offer an explanation for the single high finding of 2.13 mg. per cent.

A comparison of these data with the results reported by Borglin would seem to justify the assumption that a normal diet does not cause any appreciable variation in the serum choline content. In any case, experimental conditions must be said to be uniform if all blood samples are drawn in the morning from fasting individuals. The diet ingested on the previous day, even though very high in choline, should not have any effect on the serum choline content. Since the mean of the standard deviation of the individual determinations in duplicate summarized in table 1 is only 9.4 per cent, and since the diet does not have an effect on the serum choline content, other factors must be responsible for the variation in the values presented in table 1.

*Menstrual Cycle and Serum Choline.* When the serum choline concentrations given in table 1 are plotted as a function of the menstrual cycle, using fluctuating means of the values obtained over a period of 5 days, the stippled curve in figure 1 is obtained. The curve was constructed on the basis of fluctuating means of the values obtained over a 5-day period in order to eliminate the errors which may arise when fixing the day of the cycle. The cycle is here assumed to be 28 days long. It will be seen from the curve in figure 1 that maximum values were encountered on the 14th day, minimum values on the 26th day.

A statistical computation of the significance of the difference in serum choline concentration between the 26th and the 14th day shows that the probability of such a great difference between the two means being a chance occurrence is 1.4 ( $P = 1.4\%$ ). This means that the difference in choline concentration between the 26th and the 14th day is significant. The significance of the difference in choline concentration between the 22nd and the 26th day of the cycle ( $P$ ) is found to equal 8.4 per cent. Thus the difference is probably significant. The decrease in serum choline concentration is not significant since  $P$  equals 14.7 per cent.

In summary, therefore, decrease in choline concentration of the serum occurring between the 14th and the 26th day of the menstrual cycle has statistical significance,

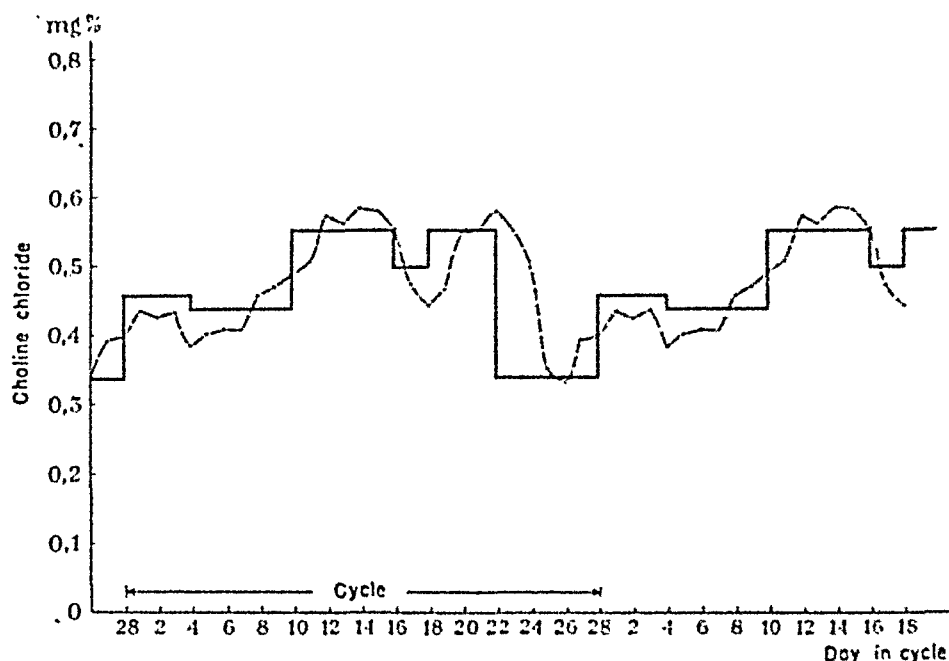


Fig. 1. CURVES indicate the serum choline content in mg. % corresponding to the different days within the menstrual cycle. Data obtained in 93 duplicate determinations. The stippled curve was plotted on the basis of fluctuating means of values obtained over a 5-day period. The solid line indicates the mean at different intervals.

whereas there is little statistical probability that the drop does not occur before the 22nd day. Accordingly, one may say that a relationship exists between the choline level of the serum and two particular phases of the menstrual cycle. The fact that a relationship is established statistically between the serum choline content and the menstrual cycle does not offer an explanation for the great variations occurring both from day to day and within the individual days of the cycle (table 1). This variability is in part attributable to the fact that conditions other than hormonal ones affect the determinations which are reported above. Among these are seasonal climatic factors.

*Seasonal Variations of Serum Choline.* In the course of further experiments it appeared that the serum choline content is subject to marked variation from season to season. The cyclic variation with the menstrual cycle is superimposed upon that of this basic annual cycle. The seasonal variation is

shown in figure 2. The height of the columns indicates the mean of the total number of choline values measured during the month. The total number of duplicate determinations made during each month is given at the bottom of the columns. No measurements are available, however, for the month of August, 1947.

It will be seen that the monthly mean levels of choline were found to be highest in the months of February and March and lowest in the months of June and July. A statistical computation of the difference between maximum and minimum values—even though based on the data of women only (13 women in July and 3 women in March)—reveals that  $P$  equals less than one per thousand, which means that the difference is highly significant.

The curve in figure 1, representing the relation of variations in choline content to phases of the menstrual cycle, was constructed on the basis of values obtained over a 12-month period. Eight determinations were made on menstruating women during the months of January, February, March and April, 1948, whereas 85 measurements were made during the remainder of the year. Accordingly, an attempt has been made to determine statistically whether or not the maximum serum choline

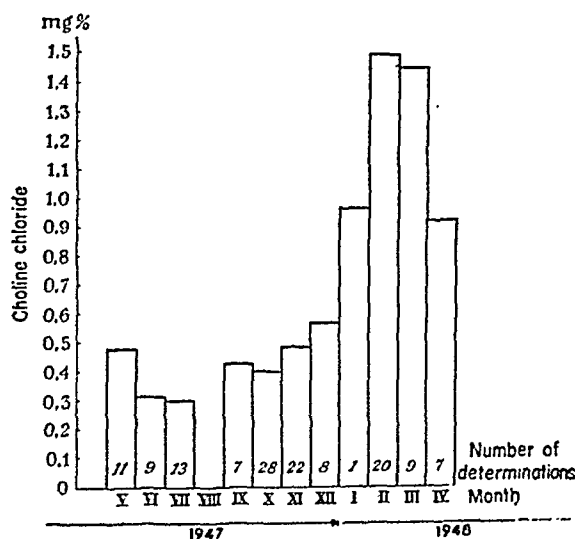


Fig. 2. SEASONAL VARIATIONS in the choline content of the serum.

level occurs about the 14th day irrespective of the season. This is so, since the value of  $P$  in each case equals 1 per cent, which means that the difference in serum choline levels between the 14th and the 26th day is still significant.

From this it may safely be concluded that a relationship clearly exists between the concentration of serum choline in the average woman and the day of her menstrual cycle. The average serum choline concentration is highest on the 14th day and lowest on the 26th day of the cycle.

#### DISCUSSION

A cyclic variation in the menstrual cycle of the choline level of the serum would appear to be correlated with corresponding hormonal changes. If so, the existence of a relationship between estrogen and choline may exist. From the following it is clear that such a correlation does hold, for serum estrogen and choline content of the serum vary together during the menstrual cycle.

Fluhmann (13) studied the estrogen content in the serum of 80 young women. It appears from his investigations that the estrogen content is highest around the 14th day and lowest around the 27th day of the menstrual cycle. Markee and Berg (14) conducted similar experiments on 75 young women. The estrogen curve given by

them shows the highest values grouped around the 14th-15th day and the lowest values around the 26th to 28th day.

In summarizing these results it seems reasonable to deduce that the concentrations of both estrogen and choline vary during the menstrual cycle in such a manner that there is a coincidence of the maxima and the minima of the two curves. This would suggest a possible relationship between estrogen and choline. The established effect of estrogen upon serum cholinesterase in rats, rabbits and guinea pigs may provide the basis for this relationship (1-4).

#### SUMMARY

Serum choline concentration is related to the menstrual cycle with the highest value around the 14th day and the lowest around the 26th. A possible relationship between choline and estrogen is pointed out. The choline content of the serum is on the average about 5 times higher in the month of February and March than in the months of June and July. (The experiments were carried out in Denmark in the period, May, 1947-May, 1948.)

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# GLYCOGENIC EFFECT OF ADRENAL CORTICAL EXTRACT

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**K**ELLEY and McDonald (1) reported alteration of the glucose tolerance of dogs exposed to simulated altitudes of 24,000 feet. During an attempt to find an explanation for this, we found that adrenal cortical extract caused a marked increase in the ability of dogs to form glycogen from glucose. A full report offering an explanation of the altered glucose tolerance is being presented (2) but it was felt that the importance of this glycogenic property of adrenal cortical extract merited its appearance in a separate paper.

## METHODS

The animals used were 6 well-trained Dalmatian coach hounds, 4 of which were litter mates. The dogs were loosely restrained in a supine position on animal boards, no anesthesia being used. Blood samples were obtained by femoral arterial puncture or by external jugular vein puncture. The dogs were fasted for 12 hours prior to the test. In experiments at a simulated altitude of 24,000 feet, the dogs were restrained prior to decompression and then decompressed at the rate equivalent to an ascent of 2000 feet per minute. This, plus previous experience of the dogs in the decompression chambers, we hoped would minimize stimulation of the sympathico-adrenal system.

The glucose tolerance test consisted of injecting intravenously 0.5 gm. of glucose/kg. of body weight, and drawing blood samples immediately before, 15 and 30 minutes, 1, 2 and 3 hours after the injection of the glucose. All blood sugar determinations were made by the method of Horvath and Knehr (3). The blood lactic acid was determined by the method of Barker and Summerson (4) and the blood pyruvic acid by the method of Friedemann and Haugen (5).

## RESULTS

The data on which this report is based are listed in table 1. The conditions under which these data were obtained, as indicated in the table headings, were during the course of standard intravenous glucose tolerance tests: *a*) at ground level (750 feet above sea level), *b*) at decompression equivalent to an altitude of 24,000 feet and beginning 1 hour after the intraperitoneal injection of 2 cc/kg. of body weight of commercial adrenal cortical extract, and *c*) at ground level at varying intervals after similar administration of the extract. Control experiments were performed giving the adrenal cortical extract without the glucose, *d*) at simulated 24,000 feet, and *e*) at ground level.

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<sup>1</sup> Captain, M.C.

TABLE I

CONDITIONS	DOG No.1	BLOOD																							
		Glucose, mg/100 cc.						Lactic Acid, mg/100 cc.						Pyruvic Acid, mg/100 cc.											
		B <sub>1</sub>		B <sub>2</sub>		Hours After B <sub>2</sub>				B <sub>1</sub>		B <sub>2</sub>		Hours After B <sub>2</sub>				B <sub>1</sub>		B <sub>2</sub>		Hours After B <sub>2</sub>			
						1/2	1	2	3					1/2	1	2	3					1/2	1	2	3
1. Standard GTT (intravenous)	1V			98		163	130	113	98		15.0		13.2	21.8	16.0	15.8		0.82		1.12	1.30	1.04	1.04		
	2V			102		157	126	118	91		24.0		17.5	17.2	12.0	11.3		1.08		0.88	1.03	0.60	0.61		
	3V			132		200	132	134	132		37.3		22.1	13.7	17.0	20.6		0.74		0.84	0.57	0.50	0.65		
	4V			118			215	162	123		14.9		13.4	19.4	13.4	13.5		0.93		0.80	0.53	0.43	0.80		
2. GTT with ACE (24,000 ft.)	2AE	88	121	124	117	161	151	127		9.6		17.5	16.9	16.4		7.3		0.88		1.28	1.22	1.04	0.99		
	3AE	96	86	84	110	66	78	70		6.0		23.0	21.8	16.5		7.8		0.58		1.41	1.74	1.06	0.73		
	4AE	116	98	78	122	102	152	143		8.6		27.8	21.7	28.9		32.0		0.96		1.84	1.56	2.16	1.92		
	2AU	91	71	130	124	122	122	122		13.6		19.2	22.4	21.0		14.0		0.71		1.12	1.17	2.16	1.51		
3. GTT with ACE, ground level	3AU	88	77	125	91	91	96	91		6.60		19.2	17.5	13.4		22.7		0.40		1.10	1.25	1.45	1.27		
	4AU <sup>2</sup>	88	75	91	80	84	96	109		10.4		21.0	17.2	14.0		35.5		0.53		1.12	1.19	1.52	2.50		
	1VE	105	225	150	147	143	143		18.6		15.3		14.5	19.4	12.2	32.0		1.54		0.91	1.16	0.58	1.30		
	2VE	89	143	135	137	135	131		12.8		15.1		13.4	19.9	15.1	36.6		1.12		0.91	0.60	0.70	2.34		
4. ACE alone at altitude	2AE <sup>1</sup>	29	102	181	152	127	127	113																	
	6AU	29	122	164	117	100	135	131																	
	5AU	96	105	164	135	122	117	124																	
	5AE	70	66	76	76		72	72		6.2		11.3		10.3		11.4		0.90		0.84			1.28		
5. ACE alone, ground level	6AE	66	82	76		104	104	104		8.4		18.6				19.2		0.64		1.52			1.63		
	2AU		96	91	91					7.30		7.11	11.6					0.97		0.46		0.71			
	3AU		91	91	88					5.16		7.23	6.60					1.13		0.51		0.40			
	4AU		89	89	83					6.20		9.54	10.4					1.14		0.63		0.51			
5. ACE alone, ground level	5AU	96	96	100	105																				
	6AU	89	83	84	122																				

B<sub>1</sub>—In altitude studies, the baseline before decompression. In all other studies, the baseline before any medication was given.

B<sub>2</sub>—Sample at the beginning of the actual or simulated Glucose Tolerance Test. GTT—Glucose Tolerance Test. ACE—Adrenal Cortical Extract.

<sup>1</sup> Dog received only part of glucose to be injected. \*V or A designates the sample being taken from the vein or the artery. U or E designates the adrenal cortical extract as being U—Upjohn's Adrenal Cortex Extract, or E—Eschaltin (Parke, Davis and Company).

<sup>2</sup> Cortical extract and glucose were injected at the same time in this animal.

Administration of 0.5 gm. of glucose/kg. of body weight to 4 of the dogs produced a minimum increment in the blood sugar of 55 mg/100 cc. at the 30-minute period. All levels returned to the baseline at the end of 3 hours. The blood lactic acid and pyruvic acid values in this test, unfortunately, were done on venous samples which later were found to be greatly affected by such minor disturbances as excitement and stasis (2). However, there were no marked increases in either throughout the test.

Administered glucose under the influence of adrenal cortical extract produced rather startling results. In the studies using the extract at a simulated altitude of 24,000 feet, the act of decompression itself produced no uniform change in the blood glucose level but, in the case of Eschatin<sup>2</sup>, the administered glucose disappeared from the blood stream within 15 minutes. In the case of Upjohn's Adrenal Cortex Extract, one dog showed rapid disappearance of the glucose while it was not markedly evident in the other one. However, in this dog, it should be noted that the blood lactic acid and pyruvic acid were greatly elevated after injection of the glucose and continued so generally throughout the test.

The added glucose at ground level also disappeared from the blood stream rapidly under the influence of adrenal cortical extract. In the case of the first two dogs, Eschatin was given only 15 minutes before the glucose. The extract itself raised the blood glucose level markedly, but the added glucose had completely disappeared from the blood stream at the end of 30 minutes. The experiment on the second dog was then repeated giving the extract and the glucose at the same time and the 15-minute increment was only 82 mg. per cent. In the case of Upjohn's Adrenal Cortex Extract, all of the injected glucose was cleared from the blood within 30 minutes in the first of two dogs and the second showed a 30-minute increment lower than expected in a normal glucose tolerance test. Urine collection for the first hour of the test in these two dogs showed reducing substances less than that equivalent to 1 gm. of glucose when titrated with Benedict's solution.

In the control studies, Eschatin was given one hour previous to decompression to a simulated altitude of 24,000 feet and very little effect on the blood glucose was evident. As can be seen from the previous experiments, Eschatin caused a rise in blood glucose 15 minutes after injection. This was quite high and presumably due to presence of some adrenalin as the glucose value at one hour was usually back to normal. Therefore, Upjohn's Adrenal Cortex Extract, which reputedly contains negligible adrenalin, was tried to ascertain its early effect. In five determinations there was no alteration of blood glucose at the 15-minute level although sometimes there was elevation at one hour.

#### DISCUSSION

In a standard glucose tolerance test, the rapidity with which the glucose disappears from the bloodstream is determined for the most part by how rapidly glycogen is formed. The formation of fat is a much slower process and injecting carbohydrate increases oxidative processes commensurate only with the increase theoretically expected from the specific dynamic action of glucose (6). Since it has been shown that

<sup>2</sup> Eschatin is an adrenal cortical extract produced by Parke, Davis and Company.

adrenal cortical extract actually promotes the conversion of both fat and protein to carbohydrate metabolites (7) and that injection of adrenal cortical extract in the hepatectomized rat causes no influence on the blood sugar (8), it follows that the rapid clearance of added glucose after the injection of adrenal cortical extract occurred because the animals had an increased ability to form glycogen.

It has been known for many years that adrenalectomy makes it difficult to maintain normal glycogen stores (9-13) particularly in the liver, unless salt and water balance are maintained carefully and the animals are well-fed (14). However, administration of adrenal cortical extract enables an adrenalectomized animal to maintain perfectly adequate glycogen stores (14, 15). Even further, giving adrenal cortical extract to normal animals causes increase to above normal glycogen deposits (16, 9). Moreover, Sundstroem and Michaels (17) found that rats kept at simulated high altitudes were able to maintain amazingly high liver glycogen levels in comparison to the nutritional state of the rest of the body as a whole. Also, the higher the altitude, the higher the glycogen content, presumably corresponding to the amount of hormones produced by the adrenal cortex. From what can be gathered from literature, many assume that all this glycogen deposition is secondary to gluconeogenesis (18). Several reports have come out, however, to support the premise that there is actually a positive effect on glycogen deposition by adrenal cortical extract. Britton and his workers actually proposed this many years ago (9), and it has been suggested as a means of assay of adrenal cortex preparations (9, 19). They however, at that time apparently did not convince everyone that adrenal cortical extract actually had glycogenic powers; but in 1940, Corey and Britton (20) perfused livers and found that adrenal cortical extract did enhance glycogen deposition in these livers. In addition to this, a preliminary report by Chiung-yun Chin and Needham (21) reported that adrenal cortical extract enhanced glycogen deposition in tissue slices from added glucose, sodium pyruvate, or DL-alanine.

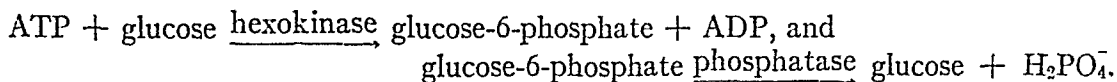
If adrenal cortical extract actually enhances glycogen deposition from glucose, then where in the metabolic cycle could it act? Glycogen deposition might be enhanced by *a*) rapid removal of inorganic phosphate, *b*) more rapid transfer of glucose through the cell membrane, *c*) any reaction which would increase the concentration of glucose-6-phosphate, or *d*) an increase in the turnover of the enzymes concerned in the reversible reactions converting glucose-6-phosphate to glycogen.

(*a*) Kalckar (23) estimates the penetration of inorganic phosphate in muscle to be approximately 1  $\gamma$  of P per minute per gram of muscle. He further gives evidence to support the premise that phosphate penetrates the cell boundary by a purely physical means. If, then, the transfer of the organic phosphate in liver cells were of the same order, the simple transfer of inorganic phosphate from the cell would be consistent with the rate of disappearance of glucose in a standard glucose tolerance test. However, inorganic phosphate must continually be used to rejuvenate the adenosine triphosphate used in the formation of glucose-6-phosphate from glucose, and Kalckar estimated the rate at which adenosine triphosphate can be rejuvenated in muscle cells to be in the order of 20 to 30  $\gamma$  of P per minute per gram of muscle, and in liver 15  $\gamma$  of P per gram of liver per minute. This provides a means for removal of phosphate much greater than is called for in a standard glucose tolerance test, so

it is unlikely that the removal of inorganic phosphate is the limiting factor determining the rapidity with which an organism can form glycogen.

(b) The transfer of glucose across the cell membrane need not be considered the limiting factor because a man doing hard physical labor may use energy to the equivalent of 100 grams of glucose per hour, which after his existing glycogen stores are depleted will be supplied largely in the form of glucose to the muscle cells. This is far above the rate needed in a normal glucose tolerance test.

(c) The concentration of glucose-6-phosphate may be increased in several ways. Since pyruvic acid concentration increases in these experiments, it would not be inhibition of any of the reversible reactions between glucose-6-phosphate and pyruvic acid; therefore, it would be 1) increase in the concentration of adenosine triphosphate, 2) decrease in the turnover of the phosphatase enzyme converting glucose-6-phosphate to glucose, or 3) increase in the turnover of hexokinase converting glucose to glucose-6-phosphate. All these possibilities can be excluded as they would all result in a decrease in blood sugar when the adrenal cortical extract was first given. In other words, the glucose-6-phosphate and glucose are at equilibrium with each other because of the equalization of the two reactions:



That this is so can be shown by giving insulin which allows a faster turnover of hexokinase (22) and a resultant fall in the blood glucose. Further, it has been shown that, although insulin may enhance glycogen deposition in normal animals with normal or low blood sugars, it enhances glycogen deposition only slightly when glucose is given to raise the blood sugar level (24).

(d) We are left, then, with the possibility of an increase in turnover of enzymes in the reversible reactions converting glucose-6-phosphate to glycogen. The end equilibrium of this system as expressed by its equilibrium constants is not changed, but the speed with which equilibrium is reached is altered.

With this in mind, consider the case where Upjohn's Cortex Extract, containing negligible amounts of adrenalin, was given alone. There was no appreciable effect on the blood glucose in the first 15 minutes. The increase in some instances by one hour would be due to the well-known gluconeogenic effect (25) of adrenal cortical extract, which is maximal about 4 hours after injection (26). Also, when adrenal cortical extract was given and the dogs were subjected to a simulated altitude of 24,000 feet, there was enough oxygen present to maintain the aerobic metabolism approximately as well as at ground level (27). So again the equilibrium was not upset, and the blood glucose was not altered appreciably until gluconeogenesis was active. On the other hand, when glucose was given intravenously before gluconeogenesis (due to the adrenal cortical extract) had been increased to a large degree, the excess glucose upset the equilibrium but disappeared from the blood stream at a rate much above that in a normal animal. The ability of the system to utilize glucose and re-establish equilibrium had increased.

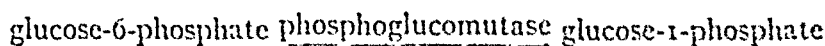
An increase in turnover of the enzymes converting glucose-6-phosphate to glycogen is entirely consistent with the observation (28, 12) that the response of adrenalectomized animals to injected adrenalin is much reduced even though their glycogen

stores are fairly high. It has further been shown that hypophysectomized animals, after about 30 days, show no hyperglycemic response to injected adrenalin even with adequate glycogen stores (29, 30). In this case, it could be possible that the adrenal cortex undergoes secondary degeneration with a reduced amount of cortical hormone resulting in a decreased turnover of the enzymes in question so that no marked hyperglycemic response occurs from the adrenalin.

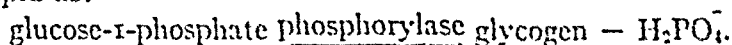
Using Cori's work on the hexokinase reaction (22), together with the effect of adrenal cortical extract on the conversion of glucose-6-phosphate to glycogen, one can easily explain the glucose tolerance of a person with Addison's disease. The intravenous glucose tolerance test of a patient with Addison's disease (31) typically shows a lower than normal peak after injection, but a greatly prolonged return to the original blood glucose level. As there are reduced amounts of adrenal cortical hormone present, the hexokinase is not inhibited so there is a rapid uptake of glucose early in the test with the formation of increased amounts of phosphorylated metabolites, resulting in a low increment due to the injected glucose. Then, since the conversion of glucose-6-phosphate to glycogen is not as rapid as when adrenal cortical hormone is present in larger amounts, it takes longer than normally to deposit glycogen and reach the original blood level.

The reaction of *dog* 2AU in the test done with adrenal cortical extract at 24,000 feet can now be explained further too. It will be noted that, early in the test, the lactic acid was increased more than that of the other dogs indicating gluconeogenesis had begun earlier and thus helped maintain the blood glucose at a higher level.

Colowick and Sutherland (32), using purified enzymes, found that the reaction *in vitro*:



was not as rapid as:



The active constituents of adrenal cortical extracts are generally accepted to be steroids; so the outcome of further work probably will be that these steroids, or a steroid, acts to activate phosphoglucomutase. An additional possibility would be a secondary change in concentration of some other factor such as magnesium ion.

#### SUMMARY AND CONCLUSIONS

Dogs previously given 2 cc/kg. of body weight of commercial adrenal cortical extract intraperitoneally were found to be able to clear their blood of intravenously injected glucose much more rapidly than without the extract. At this same time, the blood lactic acid and pyruvic acid values were elevated. Giving adrenal cortical extract or glucose in normal animals has not been shown to increase the basal metabolic rate, so the glucose must have been transformed to glycogen. Since it is known that liver glycogen stores are increased when adrenal cortical hormones are increased and that it is difficult to maintain glycogen stores when they are absent, it follows that adrenal cortical extract speeds the formation of glycogen. This must occur by increasing the turnover of the enzymes in either the reaction:

glucose-6-phosphate phosphoglucomutase glucose-1-phosphate

or

glucose-1-phosphate phosphorylase glycogen +  $\text{H}_2\text{PO}_4^-$ .

The most probable mechanism is that an adrenal cortical hormone acts as an activator to phosphoglucomutase.

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# GLUCOSE TOLERANCE OF DOGS AS ALTERED BY ATMOSPHERIC DECOMPRESSION

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**I**N A previous publication (1) it was reported that unacclimatized dogs exhibit a marked decrease in tolerance to glucose administered intravenously during acute exposure to simulated altitudes. Work has continued in an attempt to elucidate the mechanisms responsible for this phenomenon.

When attention is given to the factors responsible for determining the existing blood glucose level of an animal, the picture is seen to be extremely complex. A great number of factors come into play as there is a continuous state of flux among the great number of substances of the metabolic pool. There is constant interplay among not only substances labelled carbohydrate intermediates, but also with proteins and fats. The blood glucose level is determined by the sum total of all these reactions which are, in turn, influenced by many things; such as, concentration of the various substrates, oxygen tension, concentration of enzymes and co-enzymes, and by hormones. The adrenal hormones and insulin have a particularly marked and immediate effect. Considering the marked alteration of adrenal activity in the alarm reaction as proposed by Selye (2), it appears that the three factors likely to exert the predominant effects in altering glucose tolerance at altitude are a) the effect of low oxygen tension upon the activity of the various enzymes and the equilibria of these enzymatic systems, b) increased activity of the sympathico-adrenal system, and c) increased adrenal cortical activity. Therefore, attack of the problem consisted of an attempt to evaluate the relative importance of these three factors upon glucose tolerance.

The position of pyruvic acid and lactic acid in anaerobic glycolysis as expressed by the Embden-Meyerhof scheme has been well established. Further, the importance of pyruvic acid situated at the crossroads, so to speak, of intermediary metabolism, as an intermediary metabolite of carbohydrate, fat and protein is recognized (3). Therefore, it was reasoned that additional information could be obtained from the determination of blood lactic and pyruvic acid during the experiments.

## METHODS

The animals used in these experiments were 6 well-fed, well-trained Dalmatian coach hounds, 4 of which were litter mates. The dogs were loosely restrained in a supine position on animal boards. No anesthesia was used and blood samples were at first taken by external jugular vein puncture, but later by femoral arterial puncture. The dogs were fasted 12 hours prior to experiments. In all experiments conducted with the animals subjected to reduced pressure, a standard procedure was adhered to under which the dogs were restrained on animal boards in the low-pressure chamber and decompression to a simulated altitude of 24,000 feet was accomplished at a rate equivalent to an ascent of 2000 feet per minute. Within 5 minutes after the desired decompression was reached, the fasting blood samples were drawn. In order

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to minimize the acclimatization factor, an interval of at least one week was interposed between successive decompressions.

For the intravenous glucose tolerance test, a test dose of 0.5 gm. of glucose/kg. of body weight was given. Blood samples were drawn immediately before the glucose was given, sometimes 15 minutes after injection, and in all cases at 30, 60, 120 and 180 minutes after injection of the glucose. All blood sugar determinations were made by the method of Horvath and Knehr (3).

Blood lactic acid was determined by the method of Barker and Summerson (4) and the pyruvic acid by the method of Friedemann and Haugen (5). The precautions recommended by Friedemann and Haugen were carefully followed in the obtaining and subsequent handling of the blood samples. In the studies made in the low-pressure chamber, a portable ice container was provided so that syringes might be cold at time of use, and the precipitated samples kept refrigerated until they were analyzed.

In the experiments in which adrenalin was given, it was administered by constant intravenous drip at a rate of 40 gamma per minute throughout the entire glucose tolerance test.

#### RESULTS

The experimental results obtained with regard to blood glucose, lactic acid and pyruvic acid levels, and the lactic acid-pyruvic acid relations under various conditions are shown in table 1. The conditions under which the data were obtained are indicated in the table headings together with the experiment number. A standard glucose tolerance test was made *a*) at ground level (750 ft. above sea level), *b*) at the equivalent of 24,000 ft. altitude, *c*) at ground level during a constant infusion of adrenalin, *d*) at ground level begun 15 minutes after cessation of 3 hours of a constant infusion of adrenalin, *e*) at the equivalent of 24,000 ft. altitude beginning one hour after the intraperitoneal injection of 2 cc/kg. of body weight of adrenal cortical extract, *f*) at ground level after the injection of the same amount of extract intraperitoneally, the last two receiving their cortical extract one hour before beginning the test, the first two beginning the test 15 minutes after, and the third immediately after the cortical extract was injected, and *g*) beginning immediately after a 3-hour exposure to simulated 24,000 feet. The remainder of the experiments were simulated tests where glucose was not injected to show the response of the animal, *h*) to altitude (24,000 feet) alone, *i*) to a continuous infusion of adrenalin at ground level, *j*) to adrenal cortical extract alone at altitude (24,000 feet), and *k*) Upjohn's Adrenal Cortex Extract alone at ground level.

*Intravenous Glucose Studies.* The response of the dogs to the stress of intravenously administered glucose showed that (*experiment 1*), during the ordinary glucose tolerance test, the 30-minute level of blood glucose was at least 55 mg. above the baseline in all cases and that values had returned to the baseline by 3 hours. Lactic and pyruvic acid levels showed no significant change.

The response to glucose (*experiment 6*), at altitude, in no case showed a 30-minute increment in blood glucose as high as the minimum increment for the test

TABLE I (Continued)

CONDITIONS	DOG <sup>1</sup> NO.	BLOOD														
		Pyruvic Acid, mg/100 cc.							Lactate-Pyruvate Ratio							
		B <sub>1</sub>	B <sub>2</sub>	Hours After B <sub>2</sub>					B <sub>1</sub>	B <sub>2</sub>	Hours After B <sub>2</sub>					
				¼	½	1	2	3			¼	½	1	2	3	
1. Standard GTT (intra-venous)	1V 2V 3V 4V		0.82 1.08 0.74 0.98		1.12 0.88 0.84 0.80	1.30 1.03 0.52 0.53	1.04 0.60 0.50 0.48	1.04 0.64 0.66 0.80		18.3 22.2 50.5 15.2		16.3 19.9 26.3 16.8	16.7 16.7 26.3 36.6	15.4 20.0 34.0 28.0	15.2 17.7 31.2 23.2	
2. GTT with ACE (24,000 ft.)	2AE 3AE 4AE 2AU 3AU 4AU <sup>2</sup>	0.88 0.58 0.96 0.71 0.40 0.53	0.70 0.70 0.84 0.87 0.80 0.78	1.28 1.44 1.54 1.12 1.10 1.12	1.22 1.74 1.56 1.17 1.25 1.19	1.04 1.06 2.16 2.16 1.45 1.52		0.90 0.78 1.90 1.82 2.43 2.50	10.5 8.6 9.0 19.2 16.5 19.6	13.7 8.6 9.0 18.2 12.3 13.3	13.7 16.0 18.0 17.1 17.4 20.5	13.8 12.5 13.9 19.1 14.0 14.5	15.8 15.4 13.4 11.1 9.2 9.2		8.1 10.0 16.8 6.6 11.5 16.4	
3. GTT with ACE ground level	1VE 2VE 2AE <sup>3</sup> 6AU 5AU	1.54 1.12	1.00 1.46		0.94 0.94	1.16 0.66	0.58 0.70	1.30 2.34	12.1 11.4	18.8 10.3		15.4 14.3	9.0 16.5	20.7 18.6	24.6 15.6	
4. ACE alone at altitude	5AE 6AE	0.90 0.68	0.70 1.42		0.84 1.52			1.28 1.68	6.7 12.3	19.7 13.1		12.3			11.3 11.4	
5. ACE alone, ground level	2AU 3AU 4AU 5AU 6AU		0.97 1.13 1.14	0.46 0.51 0.68		0.71 0.40 0.53				8.0 4.6 5.5	15.5 13.9 14.0		19.2 16.5 19.6			
6. Altitude GTT (24,000 ft.)	1V 2V 3V 4V	1.25	1.29 2.70 2.60 1.10		2.44 2.56 2.70 1.98	2.48 3.84 2.34 2.48	2.07 2.78 2.76 2.54	2.47 2.74 3.46 3.58	19.0 11.1 15.1 24.1	31.6 11.1 15.1 24.1		20.2 21.1 12.6 24.9	32.6 22.2 22.3 18.1	33.8 24.0 22.9 38.6	30.1 19.7 22.7 22.4	
7. GTT under adrenalin	1V 2V 3V 4V 3A 4A		2.28 1.44 0.62 0.58 0.90 1.52		3.88 3.84 1.44 2.30 2.48 3.94	3.82 4.88 1.62 2.22 2.84 3.42	4.80 6.88 1.52 2.22 2.56 3.62	4.46 4.42 0.90 1.34 3.52 4.18		31.6 47.2 70.0 156.0 5.0 2.6			24.9 25.8 46.0 50.0 7.5 5.2	21.6 23.7 59.5 59.9 4.9 6.2	24.0 11.2 46.1 50.5 3.6 6.9	20.9 15.9 67.5 42.7 5.8 7.4
8. GTT after adrenalin	4V 3V 2A 6A	0.72 1.34 4.18 4.18	0.96 1.86 5.06 4.30		0.72 0.98 3.72 2.83	0.58 1.00 2.93 2.23	0.86 0.68 1.11 1.18	0.80 1.38 0.58 0.97	11.9 5.4 11.7 12.1	7.9 3.9 9.0 11.8		10.3 7.8 11.1 9.0	12.4 6.1 10.1 5.2	8.9 5.5 2.2 4.1	7.8 5.2 1.9 1.9	
9. GTT after altitude	3V 4V		0.46 0.90		0.70 0.78	1.04 0.68	0.64 0.58	1.36 0.40		34.8 32.2		31.0 14.1	17.5 17.6	16.9 21.4	7.4 27.2	
10. Altitude alone (24,000 ft.)	1V 2V 3V 4V 1A 2A 3A 4A 5A 6A		1.80 1.18 0.58 0.92 0.52 1.16 0.78 0.58 1.08 0.54		2.40 1.78 0.84 1.76 0.64 1.18 1.00 0.80 0.94 1.54	2.74 2.46 1.44 1.70 0.70 <sup>4</sup> 0.74 <sup>4</sup> 1.80 <sup>4</sup> 1.02 <sup>4</sup> 1.14 <sup>4</sup> 1.60 <sup>4</sup>	2.86 2.14 2.06 2.14 0.70 <sup>4</sup> 0.74 <sup>4</sup> 1.80 <sup>4</sup> 1.02 <sup>4</sup> 1.14 <sup>4</sup> 1.60 <sup>4</sup>		53.5 39.3 27.2 34.1 16.2 6.4 9.5 20.0 15.0 10.0			58.7 56.0 38.8 30.2 12.7 4.7 11.6 15.3 21.9 12.6	59.9 40.4 34.4 49.3 12.1 <sup>4</sup> 10.6 <sup>4</sup> 9.4 <sup>4</sup> 17.1 <sup>4</sup> 18.6 <sup>4</sup> 15.0 <sup>4</sup>	40.1 46.1 36.4 35.0 12.1 <sup>4</sup> 10.6 <sup>4</sup> 9.4 <sup>4</sup> 17.1 <sup>4</sup> 18.6 <sup>4</sup> 15.0 <sup>4</sup>	43.0 42.0 35.0 40.4	
11. Adrenalin alone	1V 2V 3V 4V	1.24 0.80	1.04 0.96 1.46		1.20 0.68 0.66 1.10	1.10 0.58 0.90 1.20	0.96 0.54 2.06 1.04	1.60 0.66 2.00 1.22	12.6 36.2 20.1 28.8	18.8 18.8 20.1 28.8		14.3 21.5 35.4 27.3	12.1 17.3 20.1 22.4	14.0 23.0 19.2 24.3	6.9 16.7 23.6 23.0	

<sup>1</sup> V or A designates the sample being taken from the vein or the artery. U or E designates the adrenal cortical extract as being U—Upjohn's Adrenal Cortex Extract, or E—Eschatin (Parke, Davis and Company). <sup>2</sup> Dog received only part of glucose to be injected. <sup>3</sup> Cortical extract and glucose were injected at the same time in this animal <sup>4</sup> 1½ hr. after B<sub>2</sub>.

at ground level. Except in *dog 4*, with a very flat curve, the baselinic values were never reached and two actually had begun to increase by the third hour. The lactic and pyruvic acid levels were elevated above those at ground level throughout the test, but no more than with exposure to altitude alone.

When glucose was given (*experiment 7*) with an infusion of adrenalin, the baseline was elevated a variable amount and throughout the test the blood glucose was maintained well above a normal fasting level. The dogs beginning the test with the highest baseline blood glucose values showed the least increment after administration, thus indicating that the renal threshold played some part. Blood lactic and pyruvic acids tended to be very high in both arterial and venous samples, but when arterial samples were used, the lactic acid-pyruvic acid ratios did not change much throughout the experiment.

The response to glucose (*experiment 8*), after an infusion of adrenalin, showed the 30-minute increment to be less than during an ordinary glucose tolerance test, and the baseline was usually increased. Return to the baseline blood glucose value was rapid, essentially within one hour. Lactic acid values were extremely low at the end of the experiment as was the lactic acid-pyruvic acid ratio.

The response to glucose (*experiment 2*) at altitude, under the influence of adrenal cortical extract, showed that in the case of Eschatin<sup>2</sup>, the injected glucose disappeared from the bloodstream extremely rapidly (within 15 minutes). With the Upjohn's extract, this was marked only in the case of *dog 3*, while *dog 2* offered the only example that was less marked. It should be noted that the blood lactic and pyruvic acid were very high at the one-hour level, which was not the case during the ordinary glucose tolerance test. In general, all the blood lactic acid and pyruvic acid levels were increased when glucose was injected or when the blood glucose began to show a secondary rise. *Dog 3* was interesting in that with both extracts it cleared its blood of glucose extremely rapidly with no secondary rise, but all with very high lactic and pyruvic acid levels. In all dogs, on being transferred from ground level to altitude, there was relatively little change in blood glucose.

The response to injected glucose at ground level under the influence of excess adrenal cortical extract (*experiment 3*) showed a low 30-minute increment indicating rapid clearing of the blood of glucose. The blood glucose was then either maintained or increased, so at 3 hours the level was above a normal one in all cases. In the 2 dogs on which lactic and pyruvic acids were determined, there was increase of these at the 3-hour levels.

The response to injected glucose (*experiment 9*), after exposure to 3 hours at 24,000 feet, was not markedly altered from that to glucose alone in two dogs studied.

*Test Without Injected Glucose.* Decompression to the equivalent of 24,000 feet (*experiment 10*) in only one case caused the blood glucose to go above a normal fasting level. All other cases, except one, exhibited blood glucose levels in the lower part of normal range immediately after the decompression. In all of these cases with relatively low blood glucose levels, the level gradually increased throughout the period of exposure while the two elevated ones decreased by 30-minutes to a lower

<sup>2</sup> Eschatin, an adrenal cortical extract, produced by Parke, Davis and Company.

and more normal level. No conclusions could be made about the lactic and pyruvic acid values except that they were extremely variable.

A constant infusion of adrenalin (*experiment 11*) maintained the blood glucose moderately above the baseline. Under adrenal cortical extract (*experiment 4*), decompression caused very little change in blood glucose level. Upjohn's Adrenal Cortex Extract (*experiment 5*) caused no rise in blood glucose level at the 15-minute period. In some cases, however, the 1-hour level was moderately elevated.

#### DISCUSSION

The level of blood glucose at a given time in an animal is the sum total of a great number of factors which are so great that when the blood glucose level is changed, extreme care must be exercised to ascertain just what factors have been altered. One of these, decrease in oxygen tension, has been shown to be potent in bringing about changes in the level of blood glucose (6, 7). However, there is little agreement as to its effect (6, 7); some report increase in the blood glucose levels (6, 7), some show no change, while still others show hypoglycemia. In addition to simply taking blood glucose levels, a great deal of additional information may at times be obtained by subjecting the animal to the stress of a suddenly added amount of glucose to find how it is utilized. This previously was done during exposure to simulated high altitudes and reported (1), but this in itself did not offer much in explanation of the phenomena observed.

In the reaction:  $2 \text{ reduced DPN}^3 + \text{pyruvic acid} \rightleftharpoons \text{lactic acid} + 2 \text{ oxidized DPN}$ , theoretically, the ratio of lactic acid to pyruvic acid will be determined by the ratio of reduced to oxidized DPN and therefore should reflect the level of oxygen in the tissues. This has been suggested by Friedemann and Haugen (9) who, under very carefully controlled conditions, did find small increases in humans in the lactic acid-pyruvic acid ratio at reduced atmospheric pressures.

In our experiments on dogs, we found that the same degree of significance could not be attached to the lactic acid and pyruvic acid values because of difficulty in keeping the animals quiet. This is brought out particularly when exposure to altitude alone (*experiment 10*) is studied. When venous samples were taken, there was extreme variation of both the lactic acid and pyruvic acid and lactate-pyruvate ratios throughout the test period. When arterial samples were taken, the results were much less variable but even then did not show levels significantly different from those at ground level.

At this point, it is interesting to note (*experiment 7*) the difference in results in taking venous or arterial samples when doing a glucose tolerance test and at the same time giving a constant infusion of adrenalin. Venous samples give extremely high and variable lactic acid and lactate-pyruvate ratios presumably because of the peripheral vasoconstriction and resultant stasis of the blood plus an increased glycolysis in the muscle tissue drained. The arterial samples, on the other hand, show fairly constant values for the lactate-pyruvic ratio, and the actual levels of the lactic and pyruvic acid values are what might be expected due to the added glucose and the

<sup>3</sup> Diphosphopyridinenucleotide.

glycolytic effect of adrenalin. Therefore, we have not taken credence in venous sample values but have accepted gross changes in values obtained from arterial samples.

Subjecting animals acutely to reduced oxygen tension has been shown to cause an increased production of adrenalin with a resulting increase in blood sugar (7). This effect of adrenalin occurs early; i.e., within 15 minutes after adrenalin is produced or injected. However, in our altitude experiments a gradual decompression was made to minimize the stimulation of the sympathico-adrenal system and on considering all studies at altitude, it is seldom seen that transferring the animals from the higher to the lower pressure caused an increase in the blood sugar. This is very much against the adrenal medulla playing any prominent rôle in the glucose tolerance at altitude. As can be seen from the fasting values, in all four cases where the standard glucose test was performed under decompression (*experiment 6*) the blood glucose was not elevated greatly by the decompression alone.

Consider now the remaining two effects together; namely, the effect of low oxygen tension on the various enzymes and the effect of adrenal cortical hormones. It has been shown in an associated study (10) that adrenal cortical extract enhances glycogen formation from glucose. In fact, it can be seen from *experiment 2*, table 1, that 0.5 gm. of glucose per kilogram of body weight is cleared from the bloodstream within 15 minutes. This does not rule out some smaller reduction of the action of enzymes due to lowered oxygen tension, but it is evident that at 24,000 feet, the enzymes responsible for glycogen formation from glucose are able to function rapidly and hormonal control plays much the pre-dominant rôle.

Furthermore, when the data are examined more carefully, one sees that the statement made in the previous paper (1) that the glucose tolerance of an animal during decompression is reduced is not the case. It will be noted that in no case was the 30-minute increment in blood glucose, after intravenously injected glucose, as high when the test was made at 24,000 feet as when it was made at ground level. This is also reported by Stickney, Northup and Van Liere (11). In other words, early in the test the glucose was actually taken from the blood stream more rapidly by the animal during hypoxia than when the corresponding test was done at ground level.

It has been shown that exposure to atmospheric decompression brings about stimulation of the adrenal glands with increased production of adrenal cortical hormones (12). The effect of these hormones in bringing about gluconeogenesis from protein and fat is well known (8). As can be noted from *experiment 5*, this gluconeogenic effect is evident in some cases one hour after injecting adrenal cortical extract while maximum effect has been shown to occur about the fourth hour after injection (13). Therefore, the plausible explanation of the altered glucose tolerance test performed during atmospheric decompression is that increased amounts of cortical hormones are produced by the adrenal cortex. This results in a more rapid uptake of glucose and formation of glycogen in the early stages of the test, but later gluconeogenesis from protein and fat cause the blood glucose to be raised and the increased level is prolonged. This effect can be duplicated by giving adrenal cortical extract before a glucose tolerance test at ground level and the effects of altitude are accentuated by giving the extract. It is conceivable that if the gluconeogenic effect

of adrenal cortical hormones (or extract) is somewhat delayed or not striking, that a very flat glucose tolerance curve could be obtained at altitude or after the injection of adrenal cortical extract. The data presented show that this occurred. One could go further and predict a flat glucose tolerance curve would be obtained from a well-fed animal that had been acclimatized and kept at altitude for some time. This was observed by Forbes (14) in two human subjects. This could be, because after an animal is kept at altitude for some time, the gluconeogenic effect of adrenal cortical hormones is balanced and the blood glucose returns to more normal levels (12). However, the glycogenic effect is apparently still active and Medvedeva (15) has actually isolated a factor from adrenal cortical extract which has a glycogenic effect with no effect on protein catabolism.

The blood glucose curve, after injected glucose at altitude or after injected adrenal cortical extract, varies from animal to animal depending upon individual variation in response to the glycogenic as opposed to the gluconeogenic effect of products of the adrenal cortex. For example; *dog 3*, in two tests done during atmospheric decompression after being given adrenal cortical extract, showed very little increase in his blood glucose level throughout the test even though markedly elevated lactic acid and pyruvic acid values indicated that gluconeogenesis was active. In this animal, apparently, the response to glycogenesis was more active in comparison to response to gluconeogenesis than was the case in the other animals observed. Should an animal be decompressed rapidly, excited, subjected to atmospheric decompression far beyond its tolerance or in any other way stimulated so that there is an increased production of adrenalin, then one would expect the glucose tolerance curve to be maintained at a higher level. This is probably true of *dog 1* in *experiment 6*, and also of one of the dogs in the previous paper (1).

#### SUMMARY AND CONCLUSIONS

The effect of adrenalin under various conditions was studied and indications are that stimulation of the sympathico-adrenal system plays only a minor rôle in most cases where a glucose tolerance test is performed on dogs at a simulated altitude of 24,000 feet. Apparently, there is sufficient oxygen at this altitude to allow functioning of the enzymes involved in glucose metabolism so that actually there is more rapid formation of glycogen than at ground level. The factor playing the predominant rôle is an increased production from the adrenal cortex. Although there is delayed return of the blood glucose to the baseline level after injecting glucose at a simulated 24,000 feet, careful perusal of the data presented indicates there is actually an *increase* in glucose tolerance. The injected glucose is converted to glycogen early in the test more rapidly than at ground level and the high glucose levels thereafter are due to gluconeogenesis from fat and protein brought about by the increased production of hormones by the adrenal cortex. Adrenalin may play a minor role in certain cases where the blood glucose level is maintained at markedly increased levels.

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# ELECTROLYTIC RESISTANCE OF THE BLOOD CLOT

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THREE years ago investigations were undertaken to determine objectively the strength of clot retraction. For this purpose an insulated strain gauge was introduced into fluid blood anticipating that the force of clot retraction could be measured in terms of electrical resistance. A striking increase in ohms of resistance was observed, which, if due to the pressure of the retracting clot, would have indicated an impossibly large force of about 500 pounds per square inch.

Further consideration of this result led us to the conclusion that we were measuring the electrolytic resistance of the blood clot. As a consequence, studies of this phenomenon were undertaken. Papers to be published will detail the data accumulated in the past three years of study. The present paper deals with the technique for and the principles involved in the measurement of the electrolytic resistance of the blood clot.

## METHOD

Ten ml. of blood is withdrawn slowly from the antecubital vein into a syringe which had been coated with Dri-film No. 9987.<sup>1</sup> A stop-watch is started when 5 ml. of blood are in the syringe. One ml. of blood is placed in each of two 13-mm. diameter glass tubes for the Lee-White clotting time at 37.5° C. Then 1.2 ml. of the blood is placed into each of three silicone coated glass cells for the measurement of the clot resistance (37.5° C.). Platinum electrodes are inserted into the middle of the blood sample.

The cells used to hold the blood are pyrex glass test tubes carefully selected for inside diameter within the limits of 0.447 and 0.450 inches. The test tubes are cut to an inside depth of approximately 1.050 inches. A cuff of plastic hugs the top of the cell securely (fig. 1). The cells are prepared with three coatings of Dri-film No. 9987 prior to each test.

The electrodes are constructed of platinum wire (0.020 inches thick) in the form of a circle with a horizontal cross-bar. The diameter of the circle is 0.211 or 0.172 inches (fig. 1). They are mounted on silver rods which, in turn, are mounted in a plastic cap through two holes fitted with set-screws. In this manner the electrodes can be accurately adjusted for depth and lateral position within the middle of the 1.2-ml. sample of blood. The electrode separation is 1.5 mm. The lead-in wires to the electrodes proper are first coated with one of the silicone resins (DC-804 or DC-996<sup>2</sup> and then with wax, if needed, for insulation.

The electrodes are connected into an A.C. bridge which incorporates a recording

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<sup>1</sup> Furnished through the courtesy of General Electrical Corp., Schenectady, N. Y.

<sup>2</sup> Furnished through the courtesy of the Dow-Corning Corp., Midland, Mich.

galvanometer for continuous automatic registration (fig. 2). For the initial determination of the point at which the resistance starts to leave the baseline resistance of the unclotted, fluid blood, the ratio arms of the bridge are set at 200 ohms each. This gives a full-scale deflection on the 10-inch strip chart of the recorder of 50 ohms for a maximum degree of sensitivity. For the recording of the entire clot resistance curve

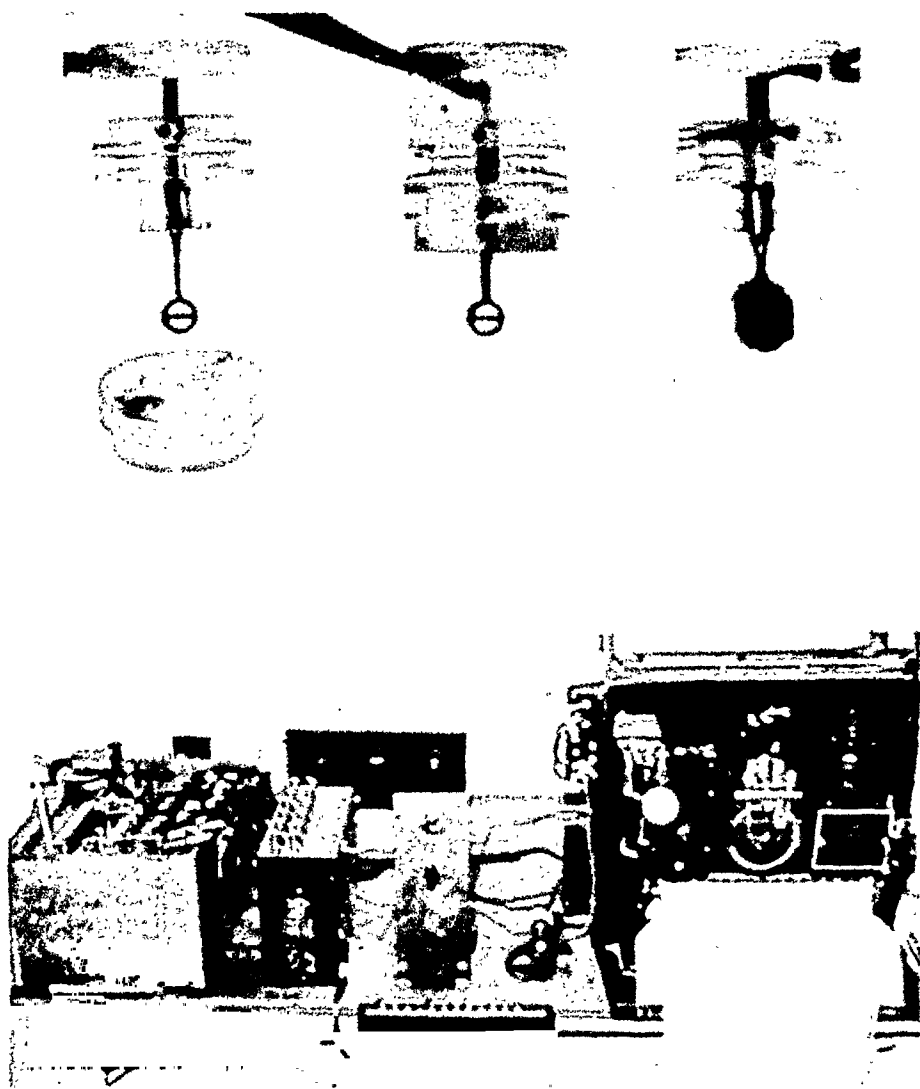


Fig. 2. *Left to right:* Water bath containing the blood cells and Lee-White tubes; telephone relay to which is connected a push-button panel for quick selection of any cell; bridge panel with bridge control knob, bridge selector switch and series resistance; recording galvanometer. In the background is a General Radio decade box which is automatically recorded on the strip chart.

over a 40- to 48-hour period, the ratio arms of the bridge are set at 5 ohms and 200 ohms to give a full-scale range of between 150 ohms and 1800 ohms. By means of a telephone relay 12 tests are recorded at one time. The instrument used in our work to date has been an old Leeds and Northrup 'pre-micromax' redesigned to use a 50-ohm slide wire and an A. C. galvanometer. A step resistance is inserted in series with the blood sample in order to place the measured resistance at a convenient place on the

strip chart, which serves the useful purpose of reducing the potential applied to the electrodes.

The bridge supply voltage used is 0.5 volts at 60 cycles. Resistance measurements at 60 cycles have been found to vary little from those at 1000 cycles. However, it is essential to platinize the electrodes quite heavily in order to avoid polarization effects which may make the initial determinations slightly unstable. No effects of the applied potential as high as several volts have been detectable.

Before the start of each experiment, the electrodes are plated with platinum black for 3 minutes and then tested in 0.01N KCl to insure resistance values that are within 3 per cent or less of each other.

The curve of resistance of the blood clot over a 40- to 48-hour period has been termed 'the electrolytic resistance curve'.

#### CRITIQUE OF METHOD

The term 'electrolytic' is used rather than electrical because the measurements are made in a conducting solution, the blood serum. The term 'resistance' is used, however, in preference to that of conductivity because what is measured is the resistance offered by the clotting and clotted blood to the passage of current through the serum.

For the purposes of the studies herein reported, the electrodes were first made 0.211 inches in diameter. Two types of electrodes were studied, the first of sheet platinum but circular, the second of wire of the design illustrated in figure 1. It was found that two factors were most important for the accurate determination of the clot resistance. These are: 1) the relationship of the size of the electrodes to the volume of the blood clot and 2) the nature of the walls of the cell in which the blood is contained. Consequently, an analysis of these two factors is included in this paper.

#### *Relationship of the Size of the Electrodes to the Volume of the Blood Clot*

This ratio is important because of the nature of the current flow between the electrodes and because the size of the blood clot varies in different clinical conditions.

a) *Current flow between the electrodes.* Since the blood clot is a poor conductor and, after clot retraction, is surrounded by serum which is highly conducting, it is essential to consider the inevitable escape of current out of the clot into the serum. This leakage of current has been observed to be a variable factor from blood to blood.

The pathways over which current flows between the electrodes consist of two parallel circuits, an internal pathway between the inner faces of the electrodes (resistance  $r$ ), and an external pathway between the outer faces of the electrodes (resistance  $r'$ ). These pathways of current flow are diagrammed for plate electrodes in figure 3.

In order to determine the resistance values of these pathways, experiments were performed with three types of circular plate electrodes (of diameter 0.211 inches): 1) with both faces of the electrodes electrically active, thus measuring the total electrolytic resistance ( $R$ ); 2) with their outer surfaces waxed, thus measuring only

the internal resistance ( $r$ ); and 3) with inner faces waxed and blocked, thus measuring only the external resistance ( $r'$ ). Comparative readings show (table 1) that the external resistance ( $r'$ ) is considerably greater than the total resistance ( $R$ ) or the internal resistance ( $r$ ). Measurements of the resistance in saline of varying concentrations show that the behavior of the electrodes in both saline and clotted blood is essentially the same, but with one distinguishing difference. The external resistance,  $r'$ , in the blood clot system is much greater in proportion to the  $R$  and  $r$  values than in the saline, indicating that the blood clot plays a significant role in the resistance observed. The  $r$  value for the blood clot is 10 times the  $R$  value, whereas the  $r'$  in saline is only three times as great as  $R$ .

The fact that a leakage of current takes place through the serum surrounding the blood clot is evidenced by the increase in resistance observed when the serum surrounding the clot is removed (table 2). This can be accomplished simply by

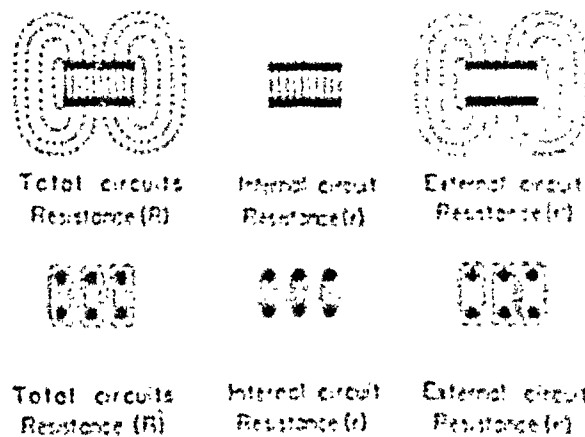


Fig. 3. *Upper:* Current flow between plate electrodes; *Lower:* current flow between wire grid electrodes.

lifting the electrodes with their clot out of the serum. As seen in table 2, the increase in resistance in the  $R$  value, the 'loss factor,' is of the order of 8 per cent to 11 per cent and for  $r$  is of the order of 5 per cent to 6 per cent, whereas the increase in resistance of the external pathway,  $r'$ , varies from 25 per cent to 57 per cent. It is evident, therefore, that a major loss in resistance occurs because of leakage of current through the serum surrounding the blood clot.

In an attempt to reduce this factor, comparable studies were made utilizing grid electrodes. The grid structure allows the current to flow around each wire (fig. 3) which provides a much shorter external pathway. In this case the 'loss factor' for  $r'$  is of the order of 9 per cent to 15 per cent as compared with the resistance loss factor of 26 per cent to 57 per cent for the plate electrodes (table 2). The changes in total measured resistance ( $R$ ) are of the order of 7 per cent to 9 per cent as compared with 8 per cent to 11 per cent for the plate electrodes where the total measured resistance ( $R$ ) was 200 ohms or less. No tests were made with conventional square electrodes, as used in other studies, as it must be immediately apparent that the corners of square electrodes of the same surface area would project closer to the surface of the blood clot, and result in larger losses of resistance. Round plate electrodes,

coated on the backs, were not considered because it was found that they furnished a poor support for the blood clot and introduced an inactive surface which could precipitate clotting.

In hundreds of routine determinations taken at various points along the course of the electrolytic resistance curve, the loss factor for  $R$  with grid electrodes was of the order of 6 per cent to 9 per cent when the resistance was approximately 400 ohms or less, and 11 per cent to 13 per cent when the resistance was between 400 and 800 ohms. (The clot volume was normal in both cases.)

*b) Ratio of 'electrode size' to blood sample volume and clot volume.* The data in (a) hold only when resistance measurements are below 800 ohms and the volume of the blood clot is normal. Clearly, reduction of the clot volume surrounding the electrodes would bring the electrodes closer to the serum and increase the loss factor; whereas increasing the clot volume would have the opposite effect. As noted initially, the

TABLE 1. COMPARATIVE VALUES OF TOTAL RESISTANCE ( $R$ ), INTERNAL RESISTANCE ( $r$ ) AND EXTERNAL RESISTANCE ( $r'$ ) FOR PLATE ELECTRODES OF DIAMETER 0.211 INCHES

TIME	IN BLOOD CLOT			SALINE CONC.	IN SALINE		
	$R$	$r$	$r'$		$R$	$r$	$r'$
<i>hr.</i>				%			
$\frac{1}{2}$	150	165	485	0.9	19	21	62
1	139	140	988	0.09	173	196	556
2	144	145	1768	0.045	339	383	1028
$2\frac{3}{4}$	148	147	1365	0.0275	672	759	2088
4	150	148	1393				
20	193	165	1445				
30	172	157	845				

blood sample volume is 1.2 ml. contained in a tube of inside diameter 0.447" to 0.450". The diameter of each electrode is 0.211". With a separation of 1.5 mm., the maximum or diagonally measured distance between the two is 0.556 cm. The volume of a theoretical sphere of this diameter is 0.0899 cu. cm. The volume of the blood sample 'sphere' having the mean diameter (0.449 inches) is 0.776 cu. cm.<sup>3</sup> The ratio of the electrode 'sphere' to the blood sample 'sphere' (0.0899 cu. cm. to 0.776 cu. cm.) is 1 to 8.6. The percentage volume of clot on the electrodes (with as many red cells as it will retain) in normal blood and in a variety of diseases, was found to be 46 per cent of the blood sample. Taking 50 per cent as an approximate figure, the ratio of the electrode 'sphere' to the clot 'sphere' is then approximately 1 to 4.3. For these ratios the error due to the 'loss factor' is, as indicated, of the order of 6 per cent to 13 per cent when the total measured resistance is under 800 ohms, using grid electrodes.

In untreated polycythemia vera it has been found that, irrespective of the red cell count, the percentage clot volume shrinks to remarkably small values of from

<sup>3</sup> The reason for taking the spherical volumes rather than the actual volumes of the blood and clot is that the current will take the shortest radial path through the clot.

accompanied by a sharp increase in resistance loss. Also, the resistance loss is greater with the plate electrodes than with the grid electrodes although the difference is significant only in the higher resistance range.

The efficiency of the electrodes appears to be measurable in yet another manner, namely, by comparing the specific resistances obtained with each type in the same (aliquot) blood sample. While the measured resistances will vary, the specific re-

TABLE 3. COMPARISON OF 'LOSS FACTOR' AND THE ELECTRODE TO CLOT VOLUME RATIOS

SUBJ.	DIAM. = 0.211"				ELEC./ CLOT RATIO	DIAM. = 0.172"				ELEC./ CLOT RATIO
	Plate elec.		Grid elec.			Plate elec.		Grid elec.		
	Ohms	% loss	Ohms	% loss		Ohms	% loss	Ohms	% loss	
M.F.			117	6.6	1/5.0			269	4.0	1/9.3
H.H.			393	14.0	1/4.8	528		978	1.5	1/8.4
F.T.			109	7.4	1/4.8	172	5.9		5.9	1/8.4
J.B.			173	11.5	1/4.6			298	4.5	1/8.6
M.G.			225	9.2	1/4.6	374	4.5	473	3.3	1/7.9
E.M.			250	7.6	1/4.6			445	5.6	1/8.6
W.D.			431	6.0	1/4.6	472	4.4			1/8.1
C.T.			229	8.4	1/4.5			265	2.9	1/8.3
E.M.			347	7.0	1/4.4			327	5.3	1/8.2
W.D.	390	8.6	385	6.7	1/4.3					
C.W.	298	12.6	410	13.6	1/4.3					
R.H.	236	6.8	251	8.5	1/4.1					
W.S.	282	8.9	265	8.6	1/4.1	374	5.9			1/7.2
A.M.	65	3.3	118	4.2	1/4.0					
D.K.			165	5.8	1/4.0	271	6.3			1/6.9
C.R.			517	9.8	1/4.0			918	10.3	1/6.9
M.N.			306	11.7	1/3.9			585	17.5	1/7.2
K.F.			528	5.2	1/3.5	1356	8.6	1942	11.3	1/6.2
K.F.	771	24.8	781	16.5	1/2.8	1304	11.9			1/5.0
K.F.	761	32.1	955	15.7	1/2.8	1248	10.1			1/4.8
A.V.			219	6.4	1/2.4			220	3.3	1/4.5
A.T.	173	13.1	241	23.8	1/2.0	461	7.3			1/3.5
C.R.			673	32.1	1/1.5			800	33.1	1/1.5
C.R.	489	24.4			1/1.1			733	23.5	1/1.9

sistances should be comparable. It will be noted in table 4 that generally the specific resistances for the smaller electrodes (both grid and plate) are greater than those for the larger electrodes. The specific resistances in the fluid, unclotted whole blood are comparable for all electrodes.

When the specific resistances obtained with the small grid electrodes are plotted against those for the large grid electrodes, the curve yields values that for the small grid are 50 per cent greater than those for the large grid. In like manner the curve for the small plate resistances against those of the large grid electrodes yields values for the plate electrodes that are 20 per cent greater. It seems reasonable to conclude, therefore, that the ratio of the size of the electrodes to that of the blood sample is of

primary importance and should not be much smaller than 1 to 8.6 and, possibly, should be of the order of 1 to 15. Also, the wire grid electrodes appear preferable to the plate electrodes of the same size partly because they yield a firmer anchor for the blood clot.

ance. Stirring of the blood prior to the introduction of the electrodes causes a marked loss in clot resistance. Should the electrodes be removed from the blood after a portion of the clot has formed and then be reintroduced, the clot resistance will level off at that point and rise no farther. The initial clot which clings to the electrodes is surrounded by a second clot that can be peeled off like the layers of an onion. However, after a clot has formed covering the electrodes, the blood may be inverted gently without disturbing the subsequent clot formation or the clot resistance rise. In addition, the redistribution of red cells so induced does not seem to change the clot resistance even where the sedimentation rate is sufficient to settle the red cells prior to complete clot formation.

When the cell wall, irrespective of the material of which it is made, is roughened

so as to induce adhesion of the clot to it, a marked reduction in clot resistance frequently occurs. Conversely, coating of the pyrex blood cell with Dri-film no. 9987, which is highly water repellant, results in a 15 per cent greater clot resistance than in the untreated glass cell (18 experiments, 54 tests). The results with the untreated cells were often erratic and in some cases the clot adhered to the cell walls. In comparative studies when the cells were made of roughened lucite, lucite coated with DC-200 fluid or Dri-film no. 9987, and glass coated with Dri-film no. 9987, the results in the glass Dri-film coated cells were almost always higher and more consistent (table 5). In addition to the above, some tests were also made of DC-802 resin, DC-995 varnish and DC-1107 on pyrex glass. These also yielded lower results and had the added disadvantage, even though they gave a permanent coat, of being easily scratched. In routine tests the Dri-film is wiped on, washed thoroughly, and then wiped dry and polished. This procedure is repeated three times prior to each test.

In order to determine the accuracy of the method, multiple tests were run on the same blood sample and on the same individual at different times. Measurements

TABLE 5. EFFECT OF VARIOUS CELL SURFACES ON ELECTROLYTIC RESISTANCE OF THE CLOT

TEST	LUCITE ROUGH	LUCITE DC-200	LUCITE DRI-FILM	GLASS DRI-FILM
1		136	192	
2	125		191	221
3	222			338
4			330	348
5			376	448

were taken at the maximum clot resistance from which the initial resistance of the fluid unclotted blood was subtracted. All tests were performed in triplicate. The deviations of the three individual measurements from the mean of the three was then taken. From the deviations so obtained in a large number of tests, the standard deviation (S.D.) was calculated.

In 128 tests in which the mean clot resistances were between 150 and 350 ohms, the S.D. so calculated was  $\pm 18.5$  ohms. In the higher resistance range, between 500 and 900 ohms, the S.D. was  $\pm 54$  ohms (46 tests). The error is greater in the higher range for reasons given. This increase in error with an increase of the clot resistance is, however, always negative, as is the loss factor upon which it presumably depends, so that the validity of any interpretations based upon an increase in resistance above normal is not impaired.

A comparison of repeated tests performed upon the same individual (normal and otherwise) performed at intervals, but while the physiological status of the subject appeared to be stable yielded data of the same order of magnitude as the agreement between triplicate determinations on the single sample of blood (table 6).

#### DISCUSSION

The technique for the determination of the electrolytic resistance of the blood clot must take into consideration certain circumstances under which the resistance is measured. These circumstances are: a) a relatively low conducting blood clot whose

ratio 1 to 8.6 showed appreciably greater losses over those of the small electrodes of ratio 1 to 15. The losses, also, were greater with the plate electrodes than with the grid electrodes.

Also of great importance is the fact that the loss of resistance is not a constant factor, but increases as the measured resistance increases. This phenomenon is dependent upon the law of parallel circuits, as expressed in the formula,  $R = r \cdot r'/r + r'$ , and on the fact that the amount of clot 'shielding' the electrodes from the serum decreases in certain instances as the clot resistance increases. In polycythemia vera the percentage clot volume becomes extraordinarily small, 12 per cent to 25 per cent as compared with the normal percentage clot volume of 46 per cent. Under these circumstances the ratio of the electrode to clot volume drops below 1 to 3, when the electrode to blood sample volume is 1 to 8.6, and large losses in resistance are encountered. When the electrodes are of ratio 1 to 15, the losses are reduced proportionately. Fortunately, these reductions in clot volume have been found almost exclusively in polycythemia vera where extremely high clot resistances are also encountered. Consequently, since the loss factor is larger only when the resistance measurements are greater the essential validity of the data is not impaired.

In addition, comparison of the specific resistances obtained with the above electrodes showed that the specific resistances are greater with the grid electrodes and when the amount of clot is large, i.e. at the ratio of 1 to 15. These variations in specific resistances also indicate that the 'loss factor' is reduced with grid electrodes and when the larger amount of clot surrounds the electrodes. Hence, it is clear that the ratio of the electrode volume to that of the blood sample should be at least as large as 1 to 8.6, and that grid electrodes are probably preferable to plate electrodes.

The above data also indicate that the specific resistance cannot be accurately calculated by the conventional formula applicable to solutions of homogeneous makeup, especially in polycythemia vera.

In recent studies (1) of the 'electrical resistance' of the blood clot, the authors use square electrodes of  $\frac{1}{4}$  sq. cm. in area, spaced 5.0 mm. apart in a blood volume of 1.5 ml. having a diameter of 12 mm. Applying the calculations used in our study, the ratio of their electrode volume to that of the blood sample volume is between 1 to 5 and 1 to 2.27 and the ratio of electrode to blood clot volume is between 1 to 2.5 and 1 to 0.64 or less. At these ratios large losses of resistance are unavoidable. In addition, the authors present their figures in terms of specific resistance. Moreover, they determine the resistance for periods of less than an hour, so that the clot resistance (at the maximum of the electrolytic resistance curve) was not determined. The specific resistance of the normal fluid blood in both their studies and ours are of the same order.

The accurate determination of the electrolytic resistance of the blood clot is also dependent upon the orderly and uninhibited retraction of the clotting blood. Experiments in which clot retraction is interfered with all show a reduction in the measured clot resistance. Roughening of the walls of the cell, thereby increasing adhesion of the blood to the cell walls, causes a variable and significant lowering of the clot resistance. This factor is enhanced when plate electrodes are used in place of grid electrodes.

# ELECTROLYTIC RESISTANCE OF THE BLOOD CLOT: RESISTANCE CLOTTING TIME, ONSET OF CLOT RETRACTION AND THE CLOT RESISTANCE

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IN THE previous paper a technique for the determination of the electrolytic resistance of the blood clot was presented (1). The present paper deals with the variations in the electrolytic resistance of the blood clot over a 40- to 48-hour period, and with the physiological significance of certain measurements of the resistance. The curve of the resistance against time will be called the electrolytic resistance curve (E.R.C.).

## PROCEDURE AND RESULTS

*Electrolytic Resistance Curve (Fig. 1).* PHASE 1. The initial rise in resistance above the baseline resistance of the fluid blood is associated with the initial precipitation of fibrin about the electrodes. The elapsed time between the taking of the blood and this point is called the resistance clotting time.

PHASE 2. There follows a rapid rise in resistance over one to two hours. This is the primary rise.

PHASE 3. Under certain conditions, a sharp break in the primary rise may be induced. This point indicates the onset of clot retraction. It is called the primary peak.

PHASE 4. Subsequent to the primary rise there is a secondary slower rise in resistance which may reach its maximum at any time within 20 hours. The maximum resistance measured minus the fluid blood resistance is the clot resistance.

PHASE 5. During the final 20- to 24-hour period a rapid fall in resistance usually occurs.

The aspects of the curve which appear to have the greatest significance are the resistance clotting time, the clot resistance and the primary peak.

As noted (1), since the specific resistances cannot be calculated, the experimental conditions under which the resistance measurements have been made must be stated. These are: 1) electrodes constructed of wire 0.020 inches thick, circular in shape of diameter 0.211 inches, with a horizontal cross-bar; 2) electrode separation of 1.5 millimeters; 3) blood sample cell of pyrex glass with an inside diameter between 0.447 and 0.450 inches, coated with Dri-film no. 9987.

*Resistance Clotting Time (R.C.T.).* The initial onset of the rise in resistance above the base-line resistance of the unclotted whole blood has been termed the resistance clotting time (R.C.T.). At this time the electrodes are coated with fine strands of fibrin whereas the surrounding blood is still fluid. As the resistance rises a clot grows

ception of clotting, was  $80.8 \pm 7.0$  ohms. In 21 normal females the fluid blood resistance was  $67.1 \pm 4.8$  (table 1). The P.E. of the difference between the two means is 1.249. The difference between the means is considerably greater than four times the P.E. so that the two means are significantly different. Since the fluid blood is a homogeneous medium the specific resistances may be calculated.<sup>1</sup>

*Clot Resistance.* From the maximum measured resistance the resistance of the fluid blood is subtracted to obtain the clot resistance (fig. 1). Considerable thought was given the question whether to record the total measured resistance or the clot resistance. In some instances no increase in resistance above that of the fluid blood is observed. In thrombocytopenic purpura, the clot resistance is extremely small. Following splenectomy the clot resistance increases but the fluid blood resistance remains unchanged. The fluid blood resistance and the clot resistance do not appear to have any direct relationship. It would appear, therefore, that the processes responsible for the clot resistance are superimposed upon those of the fluid blood resistance and are, to a greater or lesser degree, independent of them. Hence, it would seem that the clot resistance more accurately mirrors the behavior of the clot than does the total resistance.

In the normal human a marked difference in the clot resistance is observed between adult white males and females. In 21 presumably normal males between the ages of 23 and 56 years the mean clot resistance was  $311 \pm 44.4$  ohms.<sup>2</sup> In 21 normal white females between the ages of 22 and 50 years the mean clot resistance was  $179 \pm 33.5$  ohms. All these had normal menstrual periods with the exception of 2 (ages 44 and 50) who had passed their menopause and one (age 39) who had had a hysterectomy 8 years previously. In 2 female subjects the clot resistance was determined at weekly intervals over a 6- to 8-week period and in a third at 2-week intervals. There was no detectable variation in the clot resistance with the menstrual cycle as there was none in the R.C.T. The percentage clot volume in the male was 46.1 per cent and in the female, 45.7 per cent, averaging 45.9 per cent (table 1). This clot volume is of interest since the clot resistance is so different.

Considerable differences in resistance have also been found to exist in various clinical disease states. In table 2 are listed a range of unselected clot resistances and other hematological data. At first glance there would appear to be no significant correlation between either the red cell count, the platelet count, the white cell count or the percentage clot volume and the clot resistance. The highest resistances are associated with the highest red cell counts because, as will be noted later, these occur in polycythemia vera.

Of major interest is the possible existence of a relationship between the clot resistance and the concentration of platelets. From table 2 there would appear to be little relationship between these two phenomena. Of particular note are the low resistances of 115, 131, 195 and 197 ohms in association with platelet counts over 1,200,000.

However, in view of the fact that the behavior of the clotting mechanism, and

<sup>1</sup> Specific resistance = Measured resistance/K.  $K = 0.001754 \times \text{resistance of electrodes in } 0.01N \text{ KCl at } 37^\circ \text{ C.} = 0.344.$

<sup>2</sup> All  $\pm$  figures indicate standard deviations.

The mean percentage clot volume was 67.5 as compared with 46 in the normal. These observations indicate that the degree and strength of clot retraction has an important bearing on the clot resistance. Further studies of this nature will be reported later.

*The Primary Peak, a Measure of the Onset of Clot Retraction.* Early in the studies it was noted that a plateau or peak followed by a dip sometimes occurred, terminating the primary rise. This dip was of inconstant occurrence and proportions. Further

TABLE 2. CLOT RESISTANCE AND THE FORMED BLOOD ELEMENTS

EX- PERI- MENT NO.	SEX	OHMS	PLTS.	RBC (MIL)	WBC	CLOT VOL. %	EX- PERI- MENT NO.	SEX	OHMS	PLTS.	RBC (MIL)	WBC	CLOT VOL. %
180	F	977	264,000	6.72			263	M	196	599,760	4.76	12,700	44.2
110	M	908	742,800	5.45	111,300		415	F	195	2,794,000	3.40	9,900	32.5
132	M	899	438,000	6.09	8,000	23.3	125	F	192	224,000	4.77		41.7
114	M	852	584,000	7.30	17,000		102	F	188	518,000	3.81	8,750	
90	F	729	574,000	9.18	19,500		93	M	174	688,000	3.45	124,000	
95	M	613	280,000	9.34	10,250		115	F	174	276,000	4.81		
216	M	598	378,000	8.40	9,500	52.5	153	F	172	294,000	4.20		50.0
452	M	573	323,000	3.23	51,700	35.0	147	M	171	106,000	4.39	3,100	52.5
244	F	553	750,000			33.3	108	F	132	280,000	4.00		
448	M	514	557,000	5.45	23,200	40.0	259	F	131	1,200,000	3.12	20,800	39.2
194	M	433	272,680	4.00	119,000	20.0	96	M	130	323,000	4.05	5,800	
185	F	417	202,000	5.22	14,900		97	M	128	170,000	2.94	11,800	
118	F	365	210,000	4.21	11,000		113	F	126	196,000	4.56	5,100	45.8
122	F	359	536,000	4.43	13,000		262	M	125	189,000	4.50	4,000	
399	M	353	180,000	5.55	14,000	34.2	99	M	119	275,000	3.56	8,000	27.5
107	M	331	391,000	5.43	7,800		123	M	115	1,530,000	3.06	16,000	54.2
104	M	302	504,000	4.99			227	M	122	592,800	3.80	10,200	
100	M	299	398,000	4.82	8,100		103	F	114	397,000	3.61		
109	M	278	140,000	5.60	5,200		106	F	101	140,000	4.28		
411	F	276	416,000	5.20	8,000	50.0	350	M	96		3.51	27,900	33.3
121	F	216	243,000	4.50			191	F	63	164,220	3.22	5,900	41.7
246	F	197	1,733,760	3.87	46,000	33.3	289	M	7	26,260	2.54	14,200	41.7

study indicated that this was due to adventitious adhesion of the blood to the cell walls and consequent failure of the clot to retract normally around the electrodes. This peak, the primary peak (fig. 1), may be induced by causing adhesion of the blood clot to the cell walls and, hence, retraction of the clot away from the electrodes. This may be brought about by roughening the walls of the cells, by placing a fine screen around the inner walls of the cell or by similar treatment.

The primary peak may also be induced by placing the electrodes in contact with the walls of the cell. In this manner the retraction of the clot from the cell walls promptly exposes the electrodes to the highly conducting serum. Siliconing above the electrodes reduces the adhesion of the clot to them and interferes with the electrolytic conductivity only slightly.

Under both sets of conditions the clot resistance rises in characteristic manner until the primary peak is reached at which time it drops sharply. When the primary

peak is induced by roughening of the cell walls the E.R.C., depending on the strength of clot retraction, will often resume its upward trend. When the electrodes are placed at the cell walls the resistance continues downward until it approaches that of the serum with which the electrodes are in contact. The primary peak occurs before clot retraction is observed visually and never following it.

The appearance of the primary peak, when caused by adhesion of the blood clot to the cell walls, is related to the clot resistance. When the measured clot resistance is high in the neighborhood of 700 to 800 ohms, the primary peak occurs infrequently. When the clot resistance is in a middle range of about 300 to 500 ohms, the primary peak occurs fairly frequently; whereas in a lower range of resistance, below 300 ohms, the peak is almost always present. This is further evidence relating the clot resistance to the strength of clot retraction. While only a limited number of tests have as yet been performed with the second technique, it is likely that it will more consistently

TABLE 5. CLOT RESISTANCE AND CLOT VOLUME IN POLYCYTHEMIA VERA

SUBJ.	OHMS	CLOT VOL. %	SUBJ.	OHMS	CLOT VOL. %
<i>I.L.</i>	899	23.3	<i>R.G.</i> <sup>1</sup>	692	20.0
<i>M.N.</i>	825	20.8	<i>C.R.</i> <sup>1</sup>	650	15.8
<i>K.F.</i>	856	34.4	<i>A.T.</i> <sup>1</sup>	504	10.8
<i>C.R.</i>	840	36.3	<i>S.Br.</i> <sup>1</sup>	450	25.0
<i>M.T.</i>	738	15.0	<i>A.V.</i> <sup>1</sup>	238	35.8
Mean.....					23.7

<sup>1</sup> After therapy.

yield a primary peak. Since the primary peak is produced either by the adhesion of the clot to the cell walls with the first method or by the retraction of the clot away from the cell walls with the second method, it is probable that the primary peak is related to clot retraction and is an index of the onset of clot retraction.

The time of appearance of the primary peak with the first method was between 29 and 33 minutes after taking the blood. With the second method the primary peak occurred between 27 and 35 minutes after taking the blood. These observations are consistent with the visually observed clot retraction time of approximately 30 minutes. The peak is detectable within one minute, but the reliability of the technique has yet to be established. When the R.C.T. is subtracted from the primary peak time it is seen that clot retraction commences 19 to 23 minutes after the onset of fibrin formation.

The above data, although tentative, are sufficient to indicate that the primary peak is probably capable of yielding an objective and accurate measure of the onset of clot retraction.

#### DISCUSSION

The curve of resistance offered by the whole blood clot to the passage of current between platinum electrodes buried in its substance has a distinctive pattern over a

period of 40 to 48 hours. This curve, named the electrolytic resistance curve (E.R.C.), has certain characteristics which are of physiological significance.

The initial point of interest is the resistance clotting time (R.C.T.) which marks the time of onset of the rise in the E.R.C. It is associated with the initial precipitation of minute strands of fibrin upon the electrodes. Therefore, the R.C.T. would seem to determine the onset of clot formation. This is to be distinguished from those methods, such as the Lee-White, which determine the time required for a given volume of blood to clot solidly. In the light of the studies on the rate of prothrombin conversion and, hence, on the rate of fibrin formation, it would seem that the R.C.T., which measures the onset of fibrin formation, has a more specific physiological significance than those methods which measure clotting *en masse*.

Both the syringe with which the blood is obtained and the glass cell in which the blood is placed are coated with Dri-film no. 9987. This practice permits the maintenance of the fluidity of the blood for exceptionally long periods of time. The platinum electrodes introduced into the blood function as a reproducible stimulus to precipitate clotting; and the recording galvanometer furnishes an objective and highly precise indicator of the moment when the resistance starts to increase. Hence, the technique would seem to offer the most nearly ideal conditions available for the determination of a clotting time. It suffers from one serious defect, namely, that when there is no increase in resistance there is no R.C.T.

In the normal white male the R.C.T. is  $10.3 \pm 1.0$  minutes; in the normal white female the R.C.T. is  $9.5 \pm .94$  minutes. There is no statistically significant difference between these two times and the mean normal R.C.T. is 9.9 minutes. A statistically significant correlation appears to exist between the R.C.T. and the Lee-White clotting time.

During the first hour or two the resistance rises rapidly in what is termed the primary rise. In the following 20 hours there may be a smaller secondary rise during which the E. R. C. reaches its maximum and begins to decline. The maximum resistance measured from the resistance of the fluid blood as a baseline (i.e. from which the resistance of the fluid blood is subtracted) is named the clot resistance. The clot resistance itself does not appear to be related to the fluid blood resistance in any significant manner.

It is notable that the clot resistance in the normal white male and female is strikingly different, 311 and 179 ohms respectively. Thus the male clot resistance is 74 per cent greater than that of the female.

There are two conditions in which marked changes in the clot resistance are accompanied by changes in the percentage clot volume. In polycythemia vera the clot resistance is very large and the clot volume is extremely small. This is a constant and striking finding in this disease and the conclusion is inescapable that both the clot volume and the clot resistance are indications of a markedly increased strength of clot retraction.

On the other hand, in thrombocytopenic purpura a sharp reduction in clot resistance is paralleled by a large percentage clot volume, which is indicative of a poor clot retraction. Following splenectomy, the clot resistance and the clot volume both return to normal as the platelet count rises.

These observations suggest that the clot resistance is intimately related to the clot retractile function of the platelets. In the range of resistance below 300 ohms, a statistically significant correlation exists between the clot resistance and the platelet count. Unfortunately, sufficient data are not yet available in the higher ranges of resistance.

Since clot retraction functions in the production of the clot resistance advantage may be taken of this to measure the onset of clot retraction. By roughening the walls of the cell containing the blood so as to cause adhesion of the clot it is possible to interfere with the contraction of the clot about the electrodes. By placing the electrodes in contact with the walls of the cell clot retraction exposes the electrodes to the highly conductive serum. Both procedures will cause a sharply defined peak—the primary peak—of the clot resistance, which is a measure of the onset of clot retraction. In preliminary observations the onset of clot retraction occurred between 27 and 35 minutes after the taking of the blood and 19 to 23 minutes after the onset of clot formation (after the R.C.T.).

Thus the measurement of the clot resistance, which in its entirety is called the electrolytic resistance curve, yields three types of information which seem to have physiological significance; the resistance clotting time, an objective registration of the initial onset of fibrin precipitation in whole blood, the clot resistance which is strikingly different in the two sexes and is in part a consequence of clot retractile force, and the primary peak which may make possible the objective measurement of the onset of clot retraction.

#### SUMMARY AND CONCLUSIONS

The electrolytic resistance curve of the blood clot is the curve of resistance in ohms over a 40- to 48-hour period. The resistance clotting time, the onset of the rise in resistance, is associated with the initial precipitation of fibrin on the electrodes. It is, therefore, a measure of the onset of clot formation. The normal time in the adult human is 9.9 minutes.

The clot resistance is the maximum resistance of the electrolytic resistance curve measured from the resistance of the fluid blood as a base-line. It appears to be related to the strength of clot retraction in polycythemia vera and in thrombocytopenic purpura. In polycythemia vera a high clot resistance is found in association with a small percentage clot volume. This is apparently due to a greater strength of clot retraction. In thrombocytopenic purpura the reverse is true. The normal clot resistance is 311 ohms in the adult white male and 179 ohms in the adult white female. A statistically significant correlation exists between the clot resistance and the platelet count when the clot resistance was 300 ohms or lower.

The onset of clot retraction is measured by the primary peak which occurs, in preliminary studies, between 27 and 35 minutes after the blood is taken and 19 to 23 minutes after the onset of clot formation.

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# FIBRIN, A FACTOR INFLUENCING THE CONSUMPTION OF PROTHROMBIN IN COAGULATION<sup>1</sup>

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THE prothrombin consumption test as originally described (1) determines the prothrombin activity remaining in serum one hour after coagulation. By means of this test, Quick established that little prothrombin activity is lost when either platelet-poor plasma or hemophilic blood clots. These results are satisfactorily explained by postulating that in hemophilia a marked deficiency of thromboplastinogen exists so that only a small amount of prothrombin can be converted to thrombin, while in thrombocytopenia, the platelet enzyme which is required for the activation of thromboplastinogen to thromboplastin is lacking; therefore little thromboplastin can become available and consequently little prothrombin is consumed. Quick, Shanberge and Stefanini (2) have shown that the speed of prothrombin consumption increases proportionately to progressive increments of platelets, but that the final amount of prothrombin converted approaches a constant. These findings are in accord with the concept that the platelet factor is an enzyme.

Since the prothrombin consumption test offers to become an important clinical test, further study was undertaken especially to find why at times erratic results were obtained. In the course of this investigation, an observation was made which promises to provide a new insight into the mechanism of thrombosis and hemostasis. It was found that fibrin is probably the primary agent responsible for the continuous removal of thrombin in the early stage of coagulation thereby blocking and delaying the basic chain reaction which is mediated through the labilizing action of thrombin on the platelets.

## EXPERIMENTAL

The same reagents that are employed in the original one-stage method for determining prothrombin are required (3).

*Tricalcium Phosphate-treated Plasma (Calcium Phosphate Plasma).* In the determination of prothrombin in serum, fibrinogen must be supplied. This is most easily accomplished by means of oxalated plasma from which component A has been removed by means of tricalcium phosphate. This adsorbant was first introduced by Bordet and Delange (4) who concluded that it removed proserozyme, and yielded a plasma which was essentially a solution of fibrinogen. Nolf (5) in 1945 presented

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experiments which showed that prothrombin (serozyme) consisted of two substances, thrombozyme which is adsorbed by tricalcium phosphate and thrombogene which is not removed by this agent. Whether thrombogene is identical with the labile factor remains undetermined, but for practical purposes it suffices to state that tricalcium phosphate-treated plasma serves as a satisfactory agent in the determination of the residual prothrombin activity of serum.

The procedure is as follows: A measured volume of a suspension of 0.08 M tricalcium phosphate<sup>3</sup> is transferred to a test tube, 1 cc. for every cc. of oxalated plasma to be treated. The tube is centrifuged to pack the gelatinous calcium phosphates and the water is poured off. The required volume of human oxalated plasma is added and the mixture thoroughly and repeatedly stirred with a small glass rod. After 5 minutes at room temperature, the tricalcium phosphate is removed by centrifugation. The treated plasma lacks the essential prothrombin factors and therefore fails to clot after the addition of calcium chloride and thromboplastin.

*Determination of Prothrombin Activity in Serum.* To a mixture of 0.1 cc. of calcium phosphate plasma, 0.1 cc. of thromboplastin and 0.1 cc. of 0.02 M calcium chloride is added 0.1 cc. of serum by blowing from a serological pipette. The time required for the formation of a clot is accurately determined with a stop watch. It is to be emphasized that the serum must be added last. The general technique is the same as that of the prothrombin time test. The test is carried out in a water bath at 37° C. The detection of the incipient clot is best accomplished by gently tilting the test tube while holding it towards a distant source of light and observing it from underneath. All tests should be carried out in clean pyrex test tubes (13 x 100 mm.).

When it was discovered that the separation of serum from the clot influenced the prothrombin consumption, a standard technique was devised. The test tube containing the clotted blood was put in an International Clinical centrifuge and spun at full speed for exactly one minute. An additional half minute was taken to stop the centrifuge. The serum was immediately analyzed for prothrombin activity, i.e. exactly one and one-half minutes after the beginning of centrifugation.

It was realized early in the development of the prothrombin consumption test that it lacked the high degree of accuracy of the prothrombin time of oxalated plasma since serum contains varying amounts of thrombin in addition to unconverted prothrombin which becomes activated during the prothrombin time test. In the beginning of this study, no means were available to evaluate quantitatively the additive effect of thrombin on the prothrombin time. It was for this reason that in the original test, the prothrombin was determined only after the blood had been coagulated one hour since in that period it was assumed that all the formed thrombin would be removed by inactivation through the natural antithrombin, albumin-X (6).

Later it was found that more significant results were obtained if a series of clotted specimens of blood were analyzed for prothrombin after varying periods of

<sup>3</sup> The tricalcium phosphate is prepared by slowly adding with vigorous stirring 66.6 gm. of anhydrous calcium chloride dissolved in 1 liter of distilled water to an equal volume of trisodium phosphate containing 158 gm. of the anhydrous salt. After adjusting the pH to 7.0, the precipitate is repeatedly washed by decantation until only a slight trace of sodium chloride remains. The suspension is diluted to 2 liters with distilled water, making it 0.1M. From this stock suspension, a 0.008M preparation is made by diluting 8 cc. with 96 cc. of distilled water.

time. Specifically, four test tubes each containing 2 cc. of freshly drawn blood were placed in a water bath at 37° C. For normal blood the coagulation time was arbitrarily taken as 10 minutes, which is roughly the time required for 2 cc. of blood to clot at 37° C. At the end of exactly 15 minutes after coagulation, i.e. 25 minutes after the tube was placed in the water bath, the first test tube was centrifuged for one minute and the prothrombin determined immediately and after 15, 30 and 45 minutes. After 30 minutes the second tube was centrifuged and the prothrombin time determined immediately and after 15 and 30 minutes. At the end of 45 and 60 minutes after coagulation, tubes 3 and 4 respectively were centrifuged and the prothrombin determined.

*Uncorrected Prothrombin Time in Clotted Blood.* In table 1A the results on normal subjects are presented by selecting the findings of one typical subject and also a composite of results obtained on 20 healthy medical students. It will be observed that in normal individuals the prothrombin time of the serum of the first tube immediately following centrifugation averaged 8 seconds, but it lost prothrombin activity rapidly on standing. Even in the remaining tubes, the prothrombin activity of the serum immediately after centrifugation was always relatively high, but fell quickly as soon as the serum and clot were separated from intimate contact with each other.

In hemophilic blood the prothrombin time of the serum of all the tubes remained constant and centrifugation apparently had no effect, but curiously the prothrombin time of the serum was found to be invariably shorter than that of the oxalated plasma. In thrombocytopenia the prothrombin time of serum obtained after the blood had been completely coagulated and allowed to remain in the water bath for standard periods of 15, 30, 45 and 60 minutes, is, as in hemophilia, actually shorter than the 12 seconds of the oxalated plasma. Thrombocytopenic serums tend to show a gradual loss of prothrombin activity on standing.

It became increasingly more obvious as the study progressed that the speed of prothrombin consumption was strikingly affected by the separation of the serum from the clot whether brought about mechanically by centrifugation or spontaneously by clot retraction. To test whether exposure of serum might be the explanation, a comparison was made of the prothrombin consumption in a specimen of blood covered with paraffin oil with one exposed to air. The results in table 1B clearly show that such exposure has no effect on the rate or extent of prothrombin conversion.

*Effect of the Intact Unretracted Clot on the Prothrombin Consumption Test.* The evidence pointed clearly to the unretracted clot itself as the factor responsible for the delayed activation of prothrombin. Since Hirschboeck (7) had shown that no clot retraction occurs in collodion-coated vessels, the prothrombin consumption of blood coagulated in collodion-coated test tubes was studied. Surprising results were obtained. From a typical experiment presented in table 2, it can be seen that native platelet-rich plasma (obtained by the use of silicone-coated glassware) which clotted in 27 minutes showed no prothrombin consumption even after one hour following the formation of a solid clot. The serum was obtained by mechanically compressing the clot and therefore no time was lost in getting the first specimen of serum for analysis. Quickly following the expression of serum, a marked decrease of the pro-

thrombin time occurred which reached its nadir in  $3\frac{1}{2}$  minutes and then rapidly increased. These findings have a simple explanation. Immediately on freeing the serum from the clot a sudden formation of thrombin occurred, which caused the shortening of the prothrombin time, but soon the consumption of prothrombin had progressed to

TABLE 1. EFFECTS OF CENTRIFUGING AND OF EXPOSURE TO AIR ON PROTHROMBIN CONSUMPTION TIME IN CLOTTED BLOOD

	TUBE	PROTHROMBIN TIME (UNCORRECTED) OF SERUM IN SECONDS			
		Time after formation of solid clot			
		15 min.	30 min.	45 min.	60 min.
<i>A. Effect of Centrifuging</i>					
Normal	1	7½ <sup>1</sup>	22	28	32
	2		15 <sup>1</sup>	30	35
	3			23 <sup>1</sup>	33
	4				21 <sup>1</sup>
Composite of 20 normals	1	8 <sup>1</sup>	24	31	31
	2		13 <sup>1</sup>	32	35
	3			18 <sup>1</sup>	32
	4				22 <sup>1</sup>
Hemophilia	1	9 <sup>1</sup>	9	9	9
	2		8½ <sup>1</sup>	9	9
	3			9 <sup>1</sup>	9
	4				9 <sup>1</sup>
Thrombocytopenia	1	8 <sup>1</sup>	9	9½	10½
	2		9 <sup>1</sup>	10	12
	3			8 <sup>1</sup>	10
	4				8 <sup>1</sup>
<i>B. Effect of Exposure to Air</i>					
Exposed to air	1	7 <sup>1</sup>	22	24	29
	2		15 <sup>1</sup>	37	44
	3			22 <sup>1</sup>	44
Covered with oil	1	7 <sup>1</sup>	22	24	31
	2		16 <sup>1</sup>	41	50
	3			25 <sup>1</sup>	44

<sup>1</sup> Immediately after centrifugation.

such an extent that the prothrombin time of the serum increased. From this it became evident that little prothrombin was converted as long as the serum was in intimate contact with the fibrin clot, but that immediately after this contact was lost, a rapid disappearance of prothrombin occurred. To test this further the experiment was repeated in glass test tubes (table 3). Again the change in the prothrombin time immediately following the separation of serum from the clot was striking. Unfortunately, the prothrombin time of serum could not be interpreted in terms of true

prothrombin activity since the influence of the thrombin was not only indeterminable but obviously also was constantly changing since it was continuously being neutralized by the normal antithrombin, i.e. by the albumin-X.

*Prompt Arrest of Prothrombin Conversion by Sodium Citrate.* It was felt that further progress was contingent upon finding a means to separate the activity of thrombin already present in the serum from the thrombin formed from prothrombin during the prothrombin time test. The possibility that this might be accomplished by an anticoagulant such as sodium oxalate or sodium citrate was investigated. The following experiment was devised. Three test tubes each containing 2 cc. of blood from the same subject were placed in a water bath at 37° C. and allowed to clot. The clotting time was empirically fixed at 10 minutes, and 15 minutes more was allowed for the reaction to go to completion. At the end of 25 minutes, 0.2 cc. of 0.1M sodium oxalate was added to the first tube, 0.2 cc. of 0.2M sodium citrate to the second, and nothing to the third. The tubes were centrifuged for exactly one minute and an additional half-minute was required to stop the centrifuge. The prothrombin time was immediately determined. For the oxalate and citrate plasmas,

TABLE 2. PROTHROMBIN CONSUMPTION TIME FOLLOWING THE SEPARATION OF SERUM FROM CLOT OF PLATELET-RICH PLASMA<sup>1</sup> COAGULATED IN A COLLODION-COATED TEST TUBE

Time after separation of serum from clot <sup>2</sup> in minutes.....	0	2	3½	5	12	15
Prothrombin time (uncorrected) of serum in seconds.....	12	11	10	12	15	16

<sup>1</sup> Plasma was obtained from blood by employing silicone-coated glassware. <sup>2</sup> The plasma began to coagulate in 21 minutes and a rigid clot was formed in 27 minutes. One hour after coagulation appeared complete, the clot was compressed with a stirring rod to obtain serum.

0.2 cc. of 0.02M CaCl<sub>2</sub> was used. A typical result obtained with this procedure is presented in table 4.

From the findings obtained, it is clear that the objective was attained with sodium citrate. Thus, the prothrombin time of the serum to which this agent was added before centrifuging was 11 seconds and remained constant whereas the oxalated specimen was 10 seconds and the untreated blood was 6 seconds. In the latter two the prothrombin dropped, whereas it remained constant in the blood citrated before it was centrifuged. A logical explanation is that prior to centrifuging only a trace of prothrombin is changed to thrombin, but immediately on centrifuging a chain reaction is set in motion and large amounts of thrombin are quickly formed which accounts for the prothrombin time of 6 seconds. This could easily be mistaken for hyperprothrombinemia, but is merely a summation of accumulated thrombin plus thrombin formed during the prothrombin time test. When citrate is added before centrifuging, the chain reaction is not begun and the prothrombin time measures the prothrombin activity remaining in the clotted blood. The oxalated blood shows a temporary shortening followed by an increase and then becomes constant. Sodium oxalate does not stop coagulation instantaneously as Quick and Stefanini (8, 9) have repeatedly emphasized. Therefore, a certain amount of thrombin with an equivalent decrease in prothrombin occurs in the short period required for the oxalate to become completely effective. The prothrombin time of the oxalated sample

obtained immediately after centrifugation is a composite of the thrombin already formed and the quantity produced by excess thromboplastin. On standing the free thrombin is neutralized and when this occurs the prothrombin time measures the unchanged prothrombin remaining in serum.

The procedure of stopping the prothrombin conversion instantaneously with sodium citrate, then allowing the thrombin to become neutralized and analyzing the serum for unaltered prothrombin by the prothrombin time test, permits one to follow the progressive reaction occurring in blood clotted in a test tube. The actual procedure consisted in placing 2 cc. of blood in a series of test tubes and allowing them to remain in a water bath at 37° C. for 25 minutes. All the tubes were then centrifuged one minute and an additional half minute taken for stopping the centrifuge. At specific intervals of time, 0.2 cc. of 0.2M sodium citrate was added to each of the tubes consecutively. After 5 minutes, the prothrombin time of the citrated serum was determined. The prothrombin values were calculated from Quick's prothrombin curve. The striking results showing the insignificant consumption of prothrombin

TABLE 3. SPEED OF PROTHROMBIN CONSUMPTION (UNCORRECTED) OF CLOTTED BLOOD IN A GLASS TUBE FOLLOWING CENTRIFUGATION

Time after centrifugation in minutes.....	0	1	2	3	4	5	6	7	10
Prothrombin time (uncorrected) of serum in seconds.....	8	5	7	8	12	15	18	22	25

prior to centrifugation and the precipitous drop after the separation of free serum is given in figure 1.

#### DISCUSSION

It has always been tacitly accepted that when normal blood clotted in a test tube, the process was completed promptly, i.e. the fibrinogen was entirely converted to fibrin, nearly all the prothrombin became activated and the excess thrombin was mostly neutralized by the natural antithrombin, albumin-X. The results obtained in this study lead to a radically different view. Actually, so little prothrombin is changed to thrombin even 15 minutes or longer after a solid clot has formed that it is hardly measurable. It would be difficult to explain this observation by any of the older hypotheses of blood coagulation including the classical theory of Morawitz. It can, however, be readily accounted for by the new concept that a chain reaction occurs in which thrombin labilizes platelets which in turn liberate more enzyme to activate thromboplastinogen, thus accelerating the formation of more thrombin.

In the ordinary clotting of blood in a test tube, enough platelets disintegrate to activate some thromboplastin and therefore a small amount of thrombin is formed. It is quickly combined with fibrinogen for which it has a great avidity. The union is probably very transient as is the combination of an enzyme with its substrate. Very quickly a meshwork of fibrin is formed with an enormous adsorptive surface which immediately begins to remove the nascent thrombin so expeditiously and completely that little remains available for further labilization of platelets. This adsorption continues until saturation occurs, after which thrombin begins to accumulate with the result that labilization of platelets again resumes; the block to

the autocatalytic reaction is thereby removed and rapid consumption of prothrombin ensues. It is interesting that the adsorption of thrombin by fibrin has long been recognized. Howell (10) actually prepared his thrombin by extracting fibrin with 8 per cent sodium chloride solution and recently Seegers (11) has made quantitative studies of the adsorption of thrombin by fibrin.

When clot retraction takes place, the serum is no longer dispersed uniformly through the fibrin reticulum, consequently the rapid or almost instantaneous adsorption of thrombin is stopped. The autocatalytic reaction therefore is set up which explains why a rapid fall in prothrombin occurs promptly following the extrusion of serum.

The adsorption of thrombin by fibrin although clearly recognized has been entirely overlooked as a significant factor affecting coagulation and hemostasis. It has always been an enigma why the thrombosis in an area of injured tissue remains localized. Since it is well recognized that one part of thrombin can coagulate several thousand parts of fibrinogen, the potential thrombin contained in a few cc.

TABLE 4. ADDITION OF SODIUM OXALATE AND SODIUM CITRATE TO CLOTTED BLOOD PRIOR TO CENTRIFUGATION TO PREVENT CONVERSION OF PROTHROMBIN TO THROMBIN AFTER THE SEPARATION OF THE SERUM FROM THE CLOT WITH THE OBJECTIVE OF OBTAINING A TRUE VALUE OF PROTHROMBIN CONSUMPTION

<i>Time after formation of solid clot</i>	Prothrombin Consumption Time		
	15 min.	30 min.	60 min.
Normal blood + sodium oxalate 0.1M <sup>1</sup> .....	10	13½	13½
Normal blood + sodium citrate 0.2M <sup>1</sup> .....	11 <sup>2</sup>	11	11
Normal blood.....	6	26	31

<sup>1</sup> 0.2 cc. added to the 2 cc. of clotted blood.    <sup>2</sup> The prothrombin time of oxalated plasma was 11½ seconds.

of blood is enough to cause generalized thrombosis of the complete vascular system. The physiological safeguards have never been clearly defined. The existence of a plasma or serum antithrombin (albumin-X) has been generally accepted, but this agent by no means acts instantaneously; therefore it is improbable that it alone could block the chain reaction from being set into motion. In fact there is experimental evidence that it cannot. One of us (A. J. Q.) had the opportunity through the courtesy of Dr. F. H. L. Taylor and his associates at the Boston City Hospital to follow the prothrombin consumption in a child with congenital afibrinogenemia. In 5 minutes after the native platelet-rich plasma had been transferred from a silicone-coated test tube to a plain glass tube, the chain reaction was in a high state of acceleration, thus showing that the albumin-X did not neutralize the thrombin sufficiently rapidly to impede the autocatalytic reaction.

In view of the findings presented it seems clear that the fibrin clot itself becomes physiologically the most important antithrombin. Its puts, so to speak, the brake on the autocatalytic reaction of coagulation and keeps it localized to the area in which vascular damage has occurred. Albumin-X only functions as a secondary defense since it removes or inactivates thrombin relatively slowly. Paradoxically, the fibrin clot which is feared most in thrombo-embolic diseases is perhaps the most im-

portant protection against extension of thromboses beyond the area that requires the thrombi to effect hemostasis.

From the above findings one may postulate that clot retraction *in vivo* may be distinctly dangerous since the extruded serum separated from the intimate contact of the adsorptive surface of fibrin will allow thrombin to form, which immediately causes an extension of intravascular clotting. If this assumption is correct the factors that favor clot retraction increase the danger of progressive intravascular clotting. One such factor would be anemia, for the smaller the cellular bulk, the faster and more complete is the clot retraction. The second factor is a rise in the number of platelets since clot retraction is roughly proportional to the platelet count.

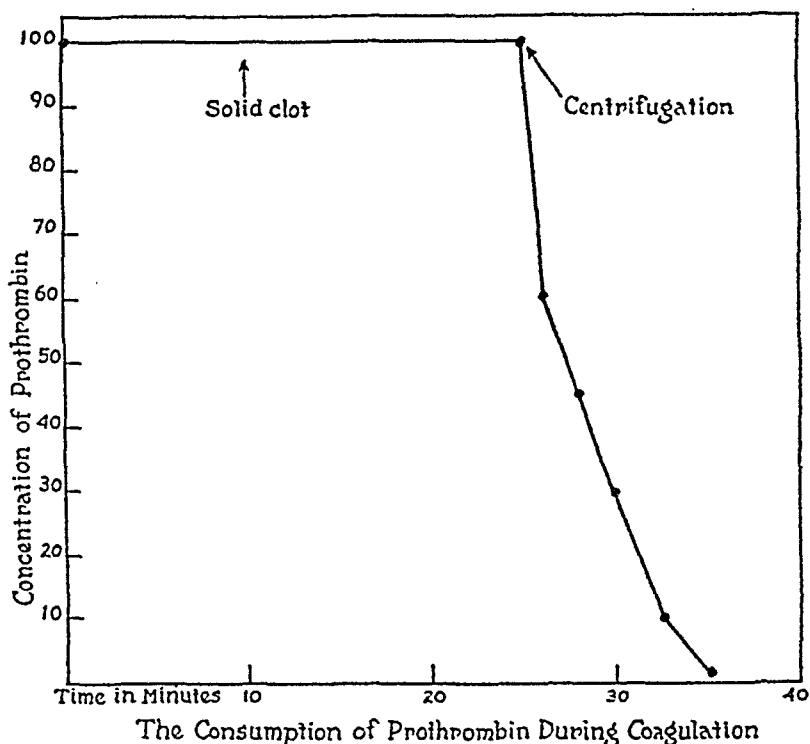


Fig. 1. PROTHROMBIN CONSUMPTION before and after the separation of the serum from the fibrin clot.

#### SUMMARY

When normal human blood is allowed to clot and to stand for one hour, more than 60 per cent of the prothrombin is usually consumed as measured by the prothrombin time of the serum. Serum obtained from clotted blood before retraction occurs has a strikingly short prothrombin time immediately after centrifugation, which quickly becomes prolonged, showing that a rapid decrease in prothrombin occurs following the separation of serum from the clot.

If sodium citrate is added to clotted blood prior to centrifugation, the serum obtained has a normal prothrombin time of 11 to 12 seconds, which indicates that the abnormally short prothrombin time of serum immediately after centrifugation is due to a summation of thrombin formed during centrifugation and the amount produced during the prothrombin time test.

The insignificant consumption of prothrombin in unretracted clotted blood is explained by the rapid and complete adsorption of the freshly formed thrombin by the fibrin clot with its large adsorptive surface that is in intimate contact with the dispersed serum. The continuous adsorption of thrombin prevents the initiation of the autocatalytic reaction of coagulation which is mediated through the labilizing action of thrombin on platelets. Fibrin appears therefore to be the most important physiological antithrombin. Its significance in hemostasis and thrombosis is discussed.

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# BEHAVIOR OF THE LEUKOCYTES OF THE RABBIT DURING PERIODS OF TRANSIENT LEUKOPENIA VARIOUSLY INDUCED

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THE profound effects of injections into dogs of extracts of *Ascaris suum* and the fluid from hydatid cysts have been described in a previous report (1). The present study is concerned chiefly with observations of the behavior of the circulating leukocytes (as seen in transparent chambers inserted in the ears of rabbits) in response to intravenous injections of the parasitic materials just mentioned and a number of other substances.

It has been known since the work of Staub and associates (2) that intravenous injections of hepatic glycogen and injections of solution of acacia cause severe leukopenia of variable duration. Rocha e Silva and associates (3) have reported inhibition of anaphylaxis in the rabbit by injections of hepatic glycogen and a polysaccharide prepared from *Ascaris lumbricoides*. They also observed almost complete disappearance of platelets from the circulating blood. They found that the leukocytes disappeared only from unanesthetized rabbits or those anesthetized with dial and ether.

Hueper (4) reported that a solution of glycogen injected intravenously into dogs in single or repeated doses caused hematic reactions characteristic of the macromolecular hematic syndrome; namely, primary transitory leukopenia, secondary myeloid leukocytosis, anemia, accelerated erythrocytic sedimentation and increased clotting time.

With the aid of transparent chambers inserted into the ears of rabbits, using the well-known technic of the Clarks, Abell and Schenck (5) observed the behavior of the minute blood vessels and that of the leukocytes during anaphylactic reactions. It was found that in a properly sensitized subject an intravenous injection of horse serum or the introduction of horse serum into the moat of a special type of chamber was followed by a marked and characteristic response of the leukocytes, which expressed itself in an increased stickiness or adhesiveness of the cells. The cells adhered so tenaciously to the endothelium of the vessels and to each other that large clumps of leukocytes were formed. These aggregations of cells frequently were of sufficient size to interfere with the flow of blood through the vessels.

## METHODS AND RESULTS

With the animals under anesthesia with pentobarbital sodium and by the use of sterile technic, transparent chambers were inserted into the ears of rabbits. When

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<sup>1</sup> Deceased. This work was begun with Dr. Graña while he was a Guggenheim Fellow working in our laboratory. The speculation and theoretical considerations must be attributed to the senior author.

the ingrowth of blood vessels had been completed, observations of the leukocytes were made with the aid of a compound microscope.

In a limited number of sensitized rabbits the interesting findings of Abell and Schenck already mentioned were seen by us after intravenous injections of horse serum.

The behavior of the leukocytes was also profoundly affected by intravenous injections of the following substances: 1) saline extracts of *Ascaris suum* in doses of 0.1 cc. for each kilogram of body weight; 2) 5 cc. of the fluid from hydatid cysts; 3) solutions of hepatic glycogen in doses of 200 mg. for each kilogram of body weight; 4) solutions of peptone; 5) 1 to 2 cc. of heparin solution (Connaught, 1000 units in each centimeter); 6) twice-normal concentrations of solutions of dried human plasma and of dried rabbit plasma; 7) 5 cc. of 6 per cent solution of acacia; 8) 1 to 5 cc. of a 6 per cent solution of a polysaccharide known as 'drolan' and also as 'dextran.'

The characteristic positive response usually began from 60 to 90 seconds after the intravenous injection of an appropriate dose of one of the effective substances.

Normally the leukocytes move haltingly along the margin of the blood stream, usually maintaining their spheroidal shape. A positive response to the substance injected is shown by a perceptible slowing. That they are becoming more adhesive is evidenced by some of them assuming an elongated or pear shape, particularly when the flow of blood through the vessel is quite rapid. When they have become sufficiently adhesive the leukocytes cease to be carried on by the most vigorous current and remain firmly attached to the endothelium. They also adhere to each other, forming large clumps that may become of such size as to interfere with the flow of blood through the vessel. It may be significant that the leukocytes adhere to the vascular endothelium and to each other but that the erythrocytes were never observed to stick to the leukocytes.

The most profound effect of all the substances used followed the injection of dextran. With most of the substances observed, the majority of the leukocytes remained out of the circulation for 15 to 20 minutes. However, injections of dextran immobilized them for 60 to 90 minutes.

On several occasions a continuous infusion of solution of acacia was given drop by drop for as long as 6 hours. The leukocytes adhered to the vessel walls while the solution was being administered, but 2 or 3 minutes after the infusion was stopped they resumed their movement.

An interesting result which is yet to be explained was obtained from experiments with dextran. Quite by accident it was discovered that after one injection of 5 cc. of dextran from which complete recovery had apparently occurred, another injection, whether immediate or long delayed, was without any observable effect. In a series of observations it was found that the leukocytes were sometimes unaffected by a second injection of dextran for as long as 4 days. In some of the experiments the leukocytes responded in a typical manner to injections of solution of acacia even though unresponsive to dextran, but in others they did not. As already indicated, the leukocytes did not show 'tachyphylaxis' when successive doses of solution of acacia were given.

From the work of previous investigators and a limited series of counts done in

our laboratory it has been shown that profound leukopenia follows the injection of glycogen and certain other substances. The leukopenia coincides with the period during which the leukocytes are observed to be adhering to the walls of the blood vessels (table 1). Consequently it is only an apparent leukopenia and is aptly termed 'transient.' An explanation of its temporary nature and an insight into its mechanism are rather clearly revealed by these findings. An explanation of why the cells become sticky has not been found.

In agreement with the reports of previous workers the number of leukocytes was greatly augmented as compared with control counts after their recovery from

TABLE 1. TYPICAL DATA ON THE NUMBER OF LEUKOCYTES IN THE CIRCULATION OF A SERIES OF RABBITS BEFORE AND AFTER AN INTRAVENOUS INJECTION OF A POLYSACCHARIDE, CALLED 'DEXTRAN'

RABBIT	DOSE OF DEXTRAN	TIME AFTER INJECTION	LEUKOCYTES PER CUBIC MILLIMETER OF BLOOD
	cc.		
1	1	Control 4 min. 60 min.	9,600 3,300 34,800
2	1	Control 6 min. 30 min.	13,200 8,400 24,400
3	3	Control 5 min. 30 min.	19,600 2,800 15,800
4	3	Control 8 min. 30 min.	9,600 3,000 15,800

adhesiveness and their return to the circulation. The leukocytosis is present for several hours.

Many other substances were injected without significant effect on the behavior of the leukocytes. This was true of injections of 1) 5 cc. of a 2 per cent solution of egg white; 2) 1 cc. for each kilogram of body weight of a 2 per cent solution of procaine hydrochloride; 3) as much as 10 cc. of a 5 per cent solution of purified gelatin in an isotonic solution of sodium chloride (Upjohn); 4) 5 to 10 cc. of isotonic saline solution, 5 cc. of 5 per cent glucose; 5) injections of various amounts of adrenal cortical extract; 6) 5 cc. of a normal concentration of human or of rabbit plasma; 7) 1 cc. of a normal concentration of horse serum in nonsensitized rabbits; and 8) 0.01 mg. of histamine for each kilogram of body weight.

#### COMMENT

It is obvious that the reactions of the leukocytes observed by Abell and Schenck during anaphylactic shock and those described in this report are fundamental in

nature. One can only speculate as to the process by which the leukocytes are made adhesive. The fact that so many different substances are able to elicit the stickiness of leukocytes emphasizes the nonspecific nature of the reaction. The transient leukopenia following injections of solutions of glycogen, acacia and such substances has given the impression that injection of substances of large molecular size elicits the phenomenon.

It has been suggested by Rocha e Silva and others that the vasculature of the liver and lungs removes the leukocytes and platelets from the circulation in anaphylaxis or after injections of glycogen, thus acting in a sense as filters. Our observations tend to support the concept that the leukocytes, after becoming adhesive, probably become attached to the endothelium in almost any region of the body and are not necessarily confined to the liver and lungs of the animal.

An insight may be gained from these observations into the mechanism involved in an inflammatory reaction. It is conceivable that an infectious process liberates leukotaxine (6) into the tissues, which finds its way into the capillaries and causes the leukocytes to become sticky and to adhere to the endothelium of the blood vessel. Eventually a large number of the cells would migrate from the blood vessel into the affected tissue with the characteristic leukocytic infiltration resulting. Abell and Schenck observed, during anaphylactic shock, the migration of many leukocytes from the vessels into the tissue spaces.

It has been found by most if not all observers that significant leukocytosis follows transient leukopenia induced by injections of such substances as glycogen. Why this should be is not at once apparent, but it might conceivably be the result of the effective operation of the mechanism that controls the number of leukocytes present in the circulating blood at any one time. Could it be that when the leukocytes become adherent to the vessel walls there is a call on the mechanism that controls the number of circulating leukocytes for more cells, which in turn would also become adherent, and thus a larger and larger number of leukocytes would be held out of circulation until released by the loss of their adhesiveness? Thereupon they would again return to the circulation in greatly augmented numbers, thus resulting in the leukocytosis so commonly observed.

#### SUMMARY AND CONCLUSIONS

Observations have been made of the behavior of the leukocytes as seen in the blood vessels that had grown into transparent chambers inserted into the ears of rabbits. The intravenous injection of extract of *Ascaris suum*, hydatid cyst fluid, solutions of acacia, glycogen and dextran, and many other substances caused the leukocytes to become sticky. They adhered to the vascular endothelium and to each other forming large clumps.

During the time when the leukocytes were adhesive, there was profound leukopenia. The duration of this phenomenon did not usually exceed 90 minutes. It may be concluded that the transient nature of the leukopenia observed in these experiments can be accounted for on the basis of the leukocytes being temporarily out of circulation as a result of their adhering to the vascular endothelium and to each other.

The possible relation of the adhesiveness of the leukocytes to certain inflammatory reactions is discussed.

The dextrone used in these experiments was kindly supplied by Dr. J. S. Lundy.

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# AGE, BODY WEIGHT AND BLOOD HYPERTENSINOGEN

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GROSSMAN and Williams (1) reported that kidneys of young rats contained more renin than those of old rats. Using preparations free of hypertensinase and other nonspecific pressor substances, it has recently been shown, (2) that kidneys of young pre-pubertal rabbits contain more renin than those of older mature rabbits.

To continue investigations concerning age and the renal-humoral mechanism the following experiments were planned to study the hypertensinogen content of blood of large, old dogs and of small, very young dogs. Pressor responses to repeated injections of purified standard hog renin<sup>1</sup> were used as a test of the blood hypertensinogen, since it has been shown by Page *et al.* (3) that as plasma renin activator (hypertensinogen) is reduced or disappears, simultaneously the pressor response to injected renin is reduced or disappears.

## PROCEDURE

The pressor responses to repeated injections of hog renin were tested in two groups of dogs. One group of 6 old animals varied in weight from 11 to 19.8 kg., and another group of 6 young animals, none older than 4 months, varied in weight from 3.2 to 5.0 kg. It was not possible to determine the exact ages of old dogs, but those selected had either lost or worn down most of their teeth and other signs of old age were apparent.

The dogs were given intraperitoneally 32.5 mg. of sodium pentobarbital per kilogram of body weight and blood pressure was recorded from the right common carotid artery with a mercury manometer. Blood pressure measurements were made 3 minutes after the renin injections, each of which consisted of 0.5 cc. containing 0.85 Goldblatt unit introduced briskly into the right femoral vein at 15-minute intervals until complete tachyphylaxis developed.

## RESULTS AND DISCUSSION

In table 1 summarized blood pressure responses to the first two renin injections and the amounts of renin necessary to develop complete tachyphylaxis are presented. The blood pressure elevations from the first renin injection in the 6 old dogs varied from 6 to 12 mm. Hg and from 4 to 9 mm. Hg to the second injection. In this same group of dogs the amount of renin necessary to induce complete tachyphylaxis

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<sup>1</sup> Kindly supplied by Dr. O. M. Helmer, Lilly Laboratories for Clinical Research, Indianapolis, Ind.

varied from 5.95 to 11.05 Goldblatt units, that is, 0.40 to 0.85 Goldblatt unit per kg. of body weight.

Blood pressure elevations from the first renin injection in the 6 young dogs varied from 24 to 32 mm. Hg and from 14 to 18 mm. Hg to the second injection. In this group of dogs the amounts of renin necessary to induce complete tachyphylaxis varied from 17.0 to 26.35 Goldblatt units, that is, 3.74 to 8.2 Goldblatt units per kg. of body weight.

Since young pre-pubertal animals have a greater supply of renin it would seem that if this renin were to function to any important degree during pubertal cardiovascular adjustments, the supply of blood hypertensinogen available should be greater also.

TABLE 1. BLOOD PRESSURE REPOSSES AND AMOUNTS OF RENIN NECESSARY TO DEVELOP COMPLETE TACHYPHYLAXIS

DOG	WEIGHT	BLOOD PRESSURE RISE		GOLDBLATT DOG UNITS OF RENIN NECESSARY TO DEVELOP COMPLETE TACHYPHYLAXIS	
		First renin injection	Second renin injection	Total	Per kg. of body wt.
	kg.	mm. Hg	mm. Hg		
1	19.8	10	6	8.5	0.43
2	18.5	9	7	11.05	0.60
3	15.0	8	6	5.95	0.40
4	14.0	12	9	7.65	0.55
5	11.0	8	4	9.35	0.85
6	11.0	6	4	8.50	0.77
7	5.0	28	14	18.7	3.74
8	4.2	32	18	17.0	4.0
9	3.8	24	16	22.1	5.8
10	3.5	29	18	17.85	5.1
11	3.4	25	14	15.3	4.5
12	3.2	27	15	26.35	8.2

Although the body mass served could be a factor determining the magnitude of pressor responses from renin in the experiments just reported, one fact alone, namely that the 6 young dogs required a greater amount of injected renin to develop complete tachyphylaxis, indicates that young, small dogs have a greater available supply of blood hypertensinogen than larger, old dogs.

#### SUMMARY

Using pressor responses to repeated injections of standard hog renin as a test, the small, young dogs studied had a greater supply of available blood hypertensinogen than large, old dogs. A possible explanation is discussed.

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# MECHANISM OF THE VASCULAR ACTION OF TETRAETHYLAMMONIUM CHLORIDE<sup>1</sup>

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**B**URN and Dale (1) some 25 years ago showed that several quaternary ammonium compounds block transmission of impulses at autonomic ganglia. Recently, tetraethylammonium chloride<sup>2</sup> (abbreviated T.E.A.) was introduced after a broad study of its pharmacology by Acheson and Moe (2) as a blocking agent which is comparatively safe, even in human beings. Since then, wide interest has been aroused, especially by Lyons and Hoobler, in the possible application of this drug, either as a test of autonomic activity or as a therapeutic chemical sympathectomy.

Acheson and Moe (2) had shown in cats and dogs that doses of from 0.1 to 10 mg/kg. body weight reduce arterial pressure, usually with diminished heart rate, the magnitude of the fall being a function of the dose. The fall was not due to action on the heart, the vascular smooth muscle, or the medullary vasomotor center, but rather from a block in the ganglia of efferent pathways of the sympathetic vasoconstrictor nerves. Further, they showed when the dose was large enough, the response was pressor, probably accompanied by discharge of adrenalin. Intra-arterial injection usually produced no change in blood flow, though occasionally decreased it without change in systemic pressure, indicating vasoconstriction.

Our interest in its relationship to hypertension arose when we found that tachyphylaxis to renin could, under certain circumstances, be overcome by large doses of the drug (3). Further, it was shown that the action of many other vasoactive drugs was greatly augmented (4, 5). During the course of these investigations, a number of other observations were made on the action of the drug itself, which aid, we hope, in understanding at least from our point of view its greatest failure, namely to select hypertensive patients for lumbar sympathectomy (6-8).

## METHOD

Except for hepatectomized dogs or those paralyzed by spinal cord section, the experiments were performed under pentobarbital anesthesia. Aseptic technique was employed for the operations with occasional exception. The animals were carefully

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<sup>2</sup> Etamon Parke, Davis and Co. We are indebted to Dr. E. C. VonderHeide for part of the drug used in this investigation.

nursed after operation, especially after destruction of parts of the nervous system. This treatment included *a*) frequent turning, *b*) emptying the bladder, *c*) fluid administration, *d*) keeping the dog clean, warm and dry, and *e*) administration of penicillin if necessary.

Methods used for determining augmentation of a variety of test drugs have already been described (9). In most of the earlier experiments, single doses of 10 mg/kg. of T.E.A. were given into the femoral vein. Later the dose was reduced to 5 mg/kg. because in some animals, as for example after hepatectomy, the larger dose usually was fatal. After cord destruction, the carotid sinus mechanism was found excessively active to such drugs as nor-adrenalin and to mechanical stimulation. Blood pressure was moderately low and the heart rate very slow. Attempts at denervating the sinus usually resulted in stoppage of the heart unless the vagi were cut first. Many animals were saved by the simple procedure of vagus section followed, rather than preceded, by exclusion of the carotid sinuses.

When inactivation of the carotid sinus mechanism is referred to it means tying off above and below the bifurcation of the carotid artery and section of both vagus nerves.

The drugs used for testing vascular responsiveness were adrenalin 0.0025 mg., nor-adrenalin<sup>3</sup> (DL arterenol, 0.05 mg.), barium chloride 0.25 cc. of solution containing 18 mg/cc., renin (0.1 cc. elevates pressure 25 to 35 min. Hg) and angiotonin 5 cat units as defined by Plentl and Page (16).

## RESULTS

*Effect of T.E.A. on Arterial Blood Pressure.* Regardless of whether pentobarbital anesthesia is used or not, the response of the blood pressure to intravenous T.E.A. (5-10 mg/kg.) is variable. The usual pattern consists of a slight rise followed by a prolonged fall, as shown by Acheson and Moe (2). The second dose augments the rise and reduces the fall and the third usually elicits a pure rise. The response is converted from depressor to pure pressor by giving repeated injections of the drug.

Tests on 94 normal anesthetized dogs showed such a wide range of response that an average figure would be almost wholly misleading. Some of these supposedly normal animals gave a pressor response after the first injection of T.E.A. without any depressor action. They responded as do animals deprived of their nervous system. Even in the same animal tested on different days, the response changes. For example, the response of one of 12 dogs studied repeatedly was -48 mm. Hg on Feb. 10, 1947; +28 mm., Feb. 20; -42, March 25; and +38, Sept. 9. This wide variability is not exceptional. Thus experiments in dogs in which a 'floor' or standard depression of arterial pressure resulting from T.E.A. is required need to be interpreted with unusual care because of this great variability. This observation confirms the variable floor found in human beings (10).

Dogs with experimental renal hypertension produced by wrapping both kidneys in silk respond to T.E.A. in the same way as normal dogs. In 6 experiments we found no consistent change in response before and after inducing severe hypertension (180-220 mmg. Hg) in the same animal. The pressure drop was the same in mm. Hg

<sup>3</sup> Dr. M. L. Tainter of Winthrop-Stearns was kind enough to supply this material.

before and after eliciting hypertension rather than the pressure reaching the same level. Since the results of this part of the investigation are negative, they are not included in this report.

*Hypotension and the Action of T.E.A.* It has been observed repeatedly that when blood pressure falls spontaneously (30–50 mm. Hg) during the course of the experiment, or directly after severe surgical operations, even though recovery of arterial pressure occurs, response to T.E.A. and to test drugs falls to low levels. Under such circumstances, administration of T.E.A. often has little effect on blood pressure, and does not increase the action of the test drugs conspicuously. Administration of large quantities of saline aids in restoring blood pressure more nearly to normal, when augmentation may appear following injection of T.E.A.

Many experiments show that the refractoriness following hypotension is not a direct function of the lowering of blood pressure. It not unusually occurs at hypertensive levels and is not immediately, if at all, relieved by T.E.A. Time, administration of fluids and maintenance of normal blood pressure levels are the factors we find important in overcoming this refractory state. After a prolonged period of hypotension and refractoriness, during which repeated injections of T.E.A. give no significant augmentation, spontaneous rise in arterial pressure usually heralds a return of responsiveness to higher levels.

*Effect of Anterior Rhizotomy.* Five successful experiments were completed in which anterior rhizotomy was performed by Dr. Robert Taylor either by one operation or in stages. The animals were allowed time for recovery. When the roots from C<sub>6</sub> to L<sub>4</sub> were included, the responses to the test drugs were uniformly increased over pre-operative tests. Administration of T.E.A. produced still further augmentation. The initial and subsequent responses to T.E.A. were pressor.

If the rhizotomy extended from D<sub>7</sub> to L<sub>2</sub> only, the initial pressor response to T.E.A. was exaggerated but the subsequent depressor component was powerful. Thus, in one dog, after this operation, T.E.A. caused a rise of 34 followed by a fall of 40 mm. Hg. But 6 days later, after extending the rhizotomy to C<sub>8</sub>, the response became purely pressor, 42 mm. Hg.

Anterior nerve root section from C<sub>7</sub> to L<sub>3</sub> produced augmentation but was inadequate as a means of replacing the full effects of T.E.A.

*'Total' Surgical Lumbo-dorsal Sympathectomy.* At different operations, the ganglia were removed from the stellate down to the fifth lumbar on both sides by Drs. Robert Taylor and Charles Devine. The left vagus nerve was sectioned two days after the right and the test made the next day. Augmentation of adrenalin response was especially prominent, while barium and renin showed little change. The response to T.E.A. was always pressor.

In a dog 20 days after completion of the sympathectomy, atropine was given to paralyze the vagus nerve endings before responsiveness was determined. Pentobarbital anesthesia was used. T.E.A., just as in the dogs with their cords destroyed, gave a pure pressor response, but augmentation was still produced though it was not great. In a third dog tested 8 days after completing the sympathectomy but without use of vagotomy or atropine, just as in the other dogs no initial depressor responses were observed. Each dose of T.E.A. exhibited only pressor action.

Thus, so-called 'total' surgical sympathectomy is an insufficient operation to

replace wholly the effects of T.E.A. in heightening the action of other drugs. It so alters the animal that depressor responses are not observed, being replaced by pressor ones.

*Effect of Cord Destruction.* In all of these nearly 100 experiments, except those in which the animal was to be killed, laminectomies and cord destruction were performed under aseptic conditions and the animals carefully nursed after the operation.

Fall in blood pressure could still be obtained on administering T.E.A. when the cord was destroyed from  $D_6$  caudad, but above this level the response became more consistently pressor. Above  $D_1$  no depressor responses and only pressor ones were observed as shown, for example, in table 1.

When a section of the cord was removed from  $C_7$  to  $D_6$  inclusive, and the animals tested 2 to 3 days later, the response was always pressor. The response to the test drugs was uniformly augmented as a result of the operation itself, but after T.E.A. it was further increased. For example, blood pressure was elevated 50 mm. Hg after

TABLE 1. EFFECT OF ACUTE PROGRESSIVE CORD DESTRUCTION ON VASCULAR RESPONSIVENESS

SUBSTANCE INJECTED	TIME	ARTERIAL PRESSURE mm. Hg	RESPONSE mm. Hg	SUBSTANCE INJECTED	TIME	ARTERIAL PRESSURE mm. Hg	RESPONSE mm. Hg
Renin	2:26	164	24	<i>Cord ligated at <math>C_6</math> and destroyed below</i>			
T.E.A., 100 mg.	2:33	168	-42	Nicotine	4:25	108	42
T.E.A., 100 mg.	2:36	134	6	Nicotine	4:31	106	44
Renin	2:38	140	50	Renin	4:34	108	62
<i>Cord ligated at <math>D_6</math> and destroyed below</i>				T.E.A.	4:42	132	34
Nicotine	3:22	142	22	Nicotine	4:45	128	0
Renin	3:27	138	16	Renin	4:47	118	60
T.E.A.	3:57	128	+4-22	Nicotine	6:50	120	-18+12
Renin	4:00	116	48	Renin	6:55	118	56
				T.E.A.	7:18	134	32
				Renin	7:22	126	54
				T.E.A.	7:44	138	36

adrenalin in one such dog before T.E.A. and 68 mm. after, when the cord had been destroyed one half hour before. But the next day, the response before was 80 mm. Hg and after T.E.A., 144 mm. Hg. T.E.A. raised the pressure 84 and 74 mm. Hg respectively.

Removal of a 3 mm. section between  $C_5$  and  $C_6$ , without destruction of the caudal portion of the cord one day before the experiment, greatly augmented the response to the test substances and T.E.A. only slightly increased it. The next day the distal cord was destroyed and assay made the following day. No significant change in responsiveness had occurred except for a return of a definite pressor response to nicotine. T.E.A. gave somewhat more augmentation than in the first experiment. In 5 other animals with the cord sectioned at  $C_6$ , the initial responses to the test drugs were surprisingly similar.

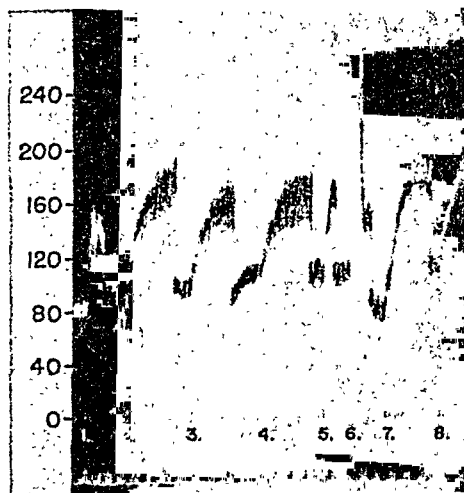
*Cord Destruction from  $C_6$  Caudal Followed by Inactivation of the Carotid Sinus Mechanism.* Laminectomy and cord destruction from  $C_6$  caudad two or more days before testing was performed on 88 animals. After the test, which was done without the necessity of anesthesia, the carotid sinuses and common carotid arteries were tied off and both vagus nerves cut (table 2).

Cord destruction alone augmented the responsiveness to the test drugs but administration of T.E.A. even further heightened the responses. Sinus exclusion and vagotomy were usually but not always followed by significant elevation of arterial pressure. Injection of T.E.A. always provoked a sharp pressor response in dogs with both cord and sinuses destroyed.

*Pithing.* Dogs under pentobarbital or other anesthesia were pithed by insertion of the pithing rod through the inner canthus of the eye. The results on vascular responsiveness were irregular. In some of the dogs the blood pressure fell to levels of from 30 to 60 mm. Hg and no augmentation to the test drugs occurred even after 6 hours. In others, responsiveness gradually increased apparently spontaneously and without benefit of T.E.A., while in still others, T.E.A. definitely aided in heightening the responses.

We assume this irregularity to be due to the different degrees of destruction of nerve tissue and of shock by the crude method used. Several years ago, we showed

Fig. 1. AUGMENTATION OF PRESSOR RESPONSE by T.E.A. in a dog with the spinal cord destroyed 2 days before from C<sub>6</sub> caudad. (1) Adrenalin, (2-4) renin, (5) T.E.A. 10 mg/kg., (6) adrenalin, (7-8) renin (no. 233). The response in a normal animal to the same amount of adrenalin is about +45 mm. Hg, renin +38 mm. Hg and T.E.A. +10-38 mm. Hg.



(11) that directly following pithing or other types of injury to the nervous system, refractoriness usually occurs for a short period.

*Effect on Bilateral Nephrectomy.* The kidneys were removed 2 days before the test in most of these experiments. Usually anesthesia was not used during the test unless the animal became unruly, when small amounts of pentobarbital were given intravenously. While the initial rise of blood pressure following T.E.A. injection seemed accentuated, the depressor component usually was present. For example, in one dog, the rise was 36 mm. Hg followed by a fall of 20 mm. Hg. The next dose raised the pressure 26 mm. followed by a fall of 4 mm. Hg. However, in many animals, only the pressor response was observed and repeated administration was accompanied by regular rises. Thirty complete experiments were performed. Augmentation of the test drugs occurred just as in normal animals.

*Bilateral Adrenalectomy.* Removal of both adrenal glands in normal dogs an hour or more before an experiment seemed to accentuate the depressor responses to T.E.A. and reduce the pressor ones. For example, in one of four such experiments, the successive doses of 5 mg/kg. of T.E.A. gave the following responses: +20 -30, +30

-10, +20 -2, +12 -16, +14, +12 -48, +18, +16 -6, +8 -14, +12 mm. Hg. The depressor response was almost constantly present despite repeated administration of the drug and the pressor was reduced.

The depressor response to T.E.A. was abolished by destruction of the cord from C<sub>6</sub> caudad, leaving the pressor response greatly heightened. Such preparations with

TABLE 2. RESPONSES IN MM. HG OF DOGS WITH CORD DESTROYED AND THE CAROTID SINUS MECHANISM INACTIVATED

	ADRENA- LIN	ADRENA- LIN	BARIUM CHLORIDE	RENIN	RENIN	T.E.A.	BLOOD PRESSURE <i>mm. Hg</i>
<i>No. 706</i>							
Control	48		44	36			88
After sinus inactivation	106		62	20		24	84
After T.E.A.	136		54	16		34	110
	140		52	48		18	102
	130			42		24	100
				58	50	8	108
			64	56	54		108
	138			48	40	40	100
	152		98	34	20		82
<i>No. 753</i>							
Control	44	54	24	62			120
After sinus inactivation	44	40	42	74			92
	84	28	18	32			68
	28	22	44				56
After 500 cc. saline	76	76		12			120
	92	86		14	24		100
				26		72	88
	94		86	14			78
<i>No. 743</i>							
2 days after cord destruction	20	28	22	36	34		112
Sinus inactivation	40		24	46			88
Next day	76		34	46	78		116
	70		36			24	128
				54		24	126
	82			40		36	116
				48	54	16	94
			36	30	52	18	92
	80			44			

the addition of bilateral nephrectomy and inactivation of the carotid sinus mechanism are therefore excellent ones to demonstrate the effects of the adrenal glands on T.E.A. response. Usually T.E.A. caused a sharp pressure elevation of 50 mm. Hg or more. But after adrenalectomy, it seemed somewhat reduced. In the different experiments, of which there were 7, the individual responses varied so much as to make strict comparison with other dogs with the adrenals intact lacking in significance. All that is safe to conclude is that in the absence of the adrenal glands, good

pressor responses are obtained on repeated administration of T.E.A. But the average response seems less than in similar preparations with intact adrenal glands. Sixteen experiments were completed.

*Hepatectomy.* The hepatectomies were performed by Dr. Ralph Prince, Dr. John J. Reinhard and Mr. William West. Several hundred such operations have now been performed. The result of this experience is that most of the dogs were in excellent condition when the testing was undertaken. We shall not at this time discuss the preparation and care of such animals despite its importance.

In these animals, injection of T.E.A. was followed by sharp fall in blood pressure and if the full dose of 10 mg/kg or even 5 mg/kg. was given, the result was usually fatal. Blood pressure fell to low levels and showed only weak tendency to recover. If doses of 2.5 mg/kg. were employed, sharp but not fatal falls occurred, which on repetition became progressively smaller. But significant pressor response did not occur, even though a total of 30 to 40 mg/kg. of T.E.A. was given. Rises of from 6 to 20 mm. Hg were observed in a few of the experiments. Thus, all of our results in the hepatectomized animal showed greatly increased sensitivity to the depressor effects of T.E.A. combined with almost complete loss of pressor effects.

*Nephrectomy, Hepatectomy, Adrenalectomy.* Since renin causes a rise in blood pressure when nephrectomy is performed just before hepatectomy (12) the possibility existed that nephrectomy might also influence the response to T.E.A. but this was not found true. Of 29 animals, 6 showed a rise of almost 20 mm. Hg when T.E.A. was administered. Adrenalectomy was then performed in 8 of these after nephrectomy-hepatectomy to determine whether this significantly affected the response. An example of this type of experiment is given in table 3. Augmented depressor effects of T.E.A. were observed as in the hepatectomized animals but possibly of somewhat greater magnitude. Pressor responses were never observed, even after repeated doses of the drug.

*Spinal Cord Destruction, Carotid Sinus Inactivation, Nephrectomy and Hepatectomy.* When the spinal cord was destroyed from C<sub>6</sub> caudad two days before the test and the carotid sinus mechanism inactivated the day of the test, the responses to T.E.A. were found to be sharply pressor with little or no depressor component. Removal of the kidneys and liver in such animals abolished the pressor response. Indeed, little response of any kind is observed to the injection of T.E.A., even though repeated doses of 5 mg/kg. were given. Occasionally, an animal exhibited a rise of 10 to 20 mm. Hg without subsequent fall.

*Administration of Priscol<sup>4</sup>, Dibenamine<sup>4</sup> and Benzodioxane to Dogs Subjected to Spinal Cord Destruction and Nephrectomy.* Spinal cord destruction and nephrectomy were usually performed a day or two before the test. Anesthesia was not necessary after the operation because of the sensory paralysis. After pressor responses to adrenalin and nor-adrenalin were ascertained, 20 mg/kg. of dibenamine was given intravenously. Thirty minutes later the responses were again determined. It was usual to find the adrenalin response either biphasic with a large depressor component, or purely depressor; while the response to nor-adrenalin was reduced to about half. This

<sup>4</sup> The Priscol was kindly furnished by Dr. Frederick Yonkman of Ciba Pharmaceutical Products, and Dibenamine by Dr. William M. Swain of Smith, Kline & French Laboratories.

confirms the observation of v. Euler (13) that nor-adrenalin is much more resistant to blocking than adrenalin. Priscol, 5 mg/kg., was then given and after a few minutes, the response was reduced from a control response of, for example, 120 mm. to 14 mm. Hg. Another dose of 2.5 to 5 mg. of Priscol blocked the nor-adrenalin response completely. Mixed solutions of Priscol (5 mg/kg.) and Benzodioxane (1 mg/kg.) were also used and found effectively to block nor-adrenalin.

TABLE 3. RESPONSE OF HEPATECTOMIZED, ADRENALECTOMIZED AND NEPHRECTOMIZED DOG<sup>1</sup>

TIME, P.M.	B.P.	ADRENA- LIN	NOR- ADRENALIN	T.E.A., 5 MG.	T.E.A.	BARIUM CHLORIDE
1:15	112	16	22	-40		
2:44	32	16	38	-28		14
3:00	52			-18	-14	
3:43	56			-10		
3:48	54		72	0		
4:02	72			4	0	
4:20	70	66				Vagotomy
4:39	114		58			Angiotonin = 8 mm. Renin = 0.
4:57	Pithed					
5:05	82		62	0		30 Angiotonin = 8 mm. Tetramethylammonium, 10 mg. = 42 mm. Renin = 0 Angiotonin = 8 mm. Obstetrical pituitrin, 2 units = 72 mm.
5:24	68	36	82			6 Angiotonin = 16 mm. Prostigmine, 0.25 mg. = 0. Ouabain, 0.25 mg. = 0. Ouabain, 0.5 mg. = 0. Sodium bicarbonate, saturated soln. 30 cc. 10.5 cc. adrenal cortex extract I.V. at 7:13 P.M. 10 cc. MgCl <sub>2</sub> · 6H <sub>2</sub> O 10 per cent I.V. at 7:55 Dose repeated
5:52	80		90			
6:16	82	44	100			
6:36	82	48	88			
6:52	82		86			
7:40	62		74			
8:02	64					
8:08			42			

<sup>1</sup> Dog 836.

Having blocked the already augmented responses to adrenalin and nor-adrenalin, T.E.A., which had previously given a striking pressor response (50-80 mm. Hg) now evoked no response or only small rises in blood pressure. As long as the response to nor-adrenalin occurred, T.E.A. could be expected to elevate blood pressure in spite of the completely reversed adrenalin response. There appeared to be a parallelism of pressor response to T.E.A. and nor-adrenalin (table 4). Nor-adrenalin, however, may produce a brisk response without necessarily a similarly good one being obtained from T.E.A.

Doses of Dibenamine and Priscol large enough to block the responses to nor-adrenalin usually reduced those to such peripherally acting substances as barium chloride and angiotonin.

*Effect of T.E.A. on the Cat's Blood Pressure and Nictitating Membrane.* Cats were anesthetized with pentobarbital, a carotid artery cannulated for blood pressure recording and the nictitating membrane attached to a writing lever. In some cases the membrane was sensitized by superior cervical ganglionectomy and in others by cocaine injection.

The initial 7 injections of 5 mg/kg., for example, produced a rise of 12 to 30 mm. Hg followed quickly by a fall of 38 mm. or more. The change to a pressor response came rather suddenly and was of the order of 44 mm. Hg. When the dose was doubled, rises of 120 mm. Hg occurred (figure 3). Responses to adrenalin, nor-adrenalin and barium chloride were greatly augmented.

During the period when arterial pressure fell as the result of T.E.A. injection, the nictitating membrane relaxed and continued to do so for some time after pure

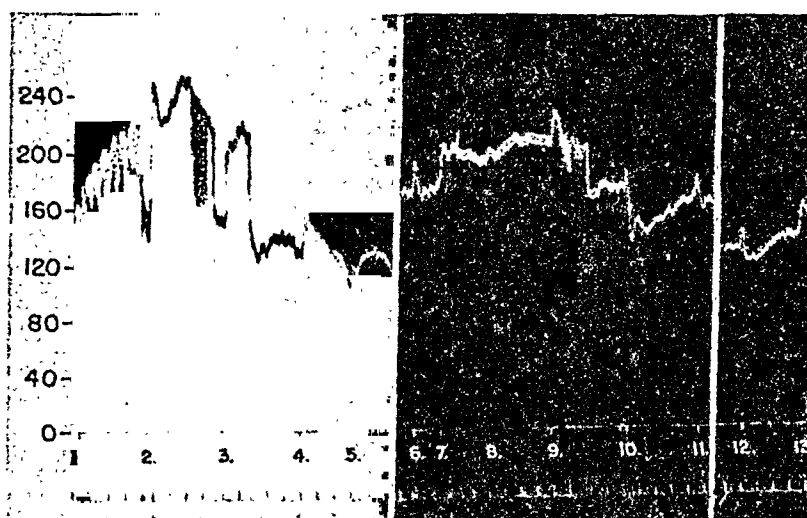


Fig. 2. PARALLEL RESPONSE of nor-adrenalin and T.E.A. 3 minutes after treatment of a nephrectomized dog with spinal cord destroyed ( $C_6 \downarrow$ ) with Priscol and Dibenamine. (1) Nor-adrenalin, (2) T.E.A. 5 mg/kg., (3) nor-adrenalin, (4) T.E.A., (5-6) nor-adrenalin, (7) barium chloride, (8) renin, (9) T.E.A., (10) adrenalin, (11-12) nor-adrenalin, (13) T.E.A. (no. 833).

pressor responses were elicited. Then a change occurred and only contraction was observed of the same order as that resulting from injection of nor-adrenalin (fig. 3). For example, equipressor amounts of adrenalin, nor-adrenalin, T.E.A. and angiotonin caused the membrane to contract 15, 8, 5 and 10 mm.

No relaxation was observed after T.E.A. in the denervated membrane but contraction occurred when the depressor action on the blood pressure was over. Injection of 2.5 mg/kg. of Priscol abolished the response of the membrane to adrenalin, nor-adrenalin and T.E.A.

*Pressor Response to T.E.A. in Patients with Cerebral Injury.* Two patients, one dying of cerebral hemorrhage, the other with cerebral thrombosis, both exhibited pressor responses even on the initial injection of T.E.A. and without significant change in heart rate. As table 5 shows, some augmentation to barium chloride and angiotonin was observed. This was not great, probably due to the fact that the autonomic system had already been largely inactivated by the cerebral thrombosis. Thus, pressor responses to T.E.A. are observed in human beings as well as in dogs, after injury of the nervous system.

## DISCUSSION

The evidence gathered by Acheson, Moe, Lyons, Hoobler and others strongly supports the view that the only significant action of T.E.A. is blocking transmission at ganglionic levels releasing the arteriolar tone due to sympathetic impulses. Blood pressure is lowered as a result and will continue to be lowered as long as there is continuing vasoconstrictor discharge from the spinal cord. With larger doses of T.E.A., the depressor action disappears, to be replaced by a pressor one.

In a few of our supposedly normal dogs, only pressor responses were obtained from the time injections were begun. Thus depressor responses, while being the usual initial ones, are not the only ones.

TABLE 4. EFFECT OF PRISCOL AND DIBENAMINE ON PRESSOR RESPONSE TO T.E.A. IN NEPHRECTOMIZED DOGS WITH SPINAL CORD DESTROYED

EXPER. NO.	ADRENALIN	NOR-ADRENALIN	T.E.A., 5 mg/kg.	DIBENAMINE, 50 mg.	PRISCOL, 5 mg/kg.	BLOOD PRESSURE, mm. Hg
8-30	84	116	36			100
				+12-14	36	110
	-12	22		+4-18	0	114
				38		110
		18	6			72
	-40	8	4			108
	-50	30	20			100
		38	18			114
		22	10			78
			100 mg.			
8-33	112	106			14	128
	-24	44		8	6	120
		48		14	0	170
		32		26	0	160
		64	96			160
		72	34			124
		26	22			212
	+10-40	12	0			170
		22	4			150
	-46	30	12			124

If the autonomic ganglia are denervated or destroyed, by anterior rhizotomy, 'total' surgical sympathectomy or cord destruction, the depressor response fails to occur and is replaced wholly by a pressor one. It was one of the purposes of this paper to attempt the explanation of this interesting change, which is not unusual in the case of several other vasoactive drugs as well.

The phenomenon is not limited to dogs, for it occurred in two patients, one suffering from cerebral hemorrhage and the other from cerebral thrombosis. Neither of these patients showed any depressor response despite the fact that their arterial pressures were not low. Had they been excessively low, it might have been supposed that the cerebral lesion had abolished or diminished sympathetic vascular tone.

Since intra-arterial injection of T.E.A. causes no change in blood flow (2) there seems to be little or no direct action on arteriolar muscle to explain its pressor action. However, this observation must be qualified because Collins (14) found T.E.A.

capable of causing contraction of the isolated terminal ileum and, further, that it increases the responses to angiotonin and histamine. From the few measurements available, there appears to be insufficient change in cardiac output to account for the pressor effect. Some more indirect mechanism is thus suggested for which explanation must be sought.

Our results were for the most part obtained on dogs but some have been from a few cats. The same reversal of T.E.A. action was observed and in many cases more striking than in dogs. The nictitating membrane continued to show some relaxation well after pressor responses were obtained suggesting that the blood pressure is not the best indicator of complete autonomic blockade. Contraction of the membrane replaces relaxation very quickly and, in degree, more closely follows that produced by

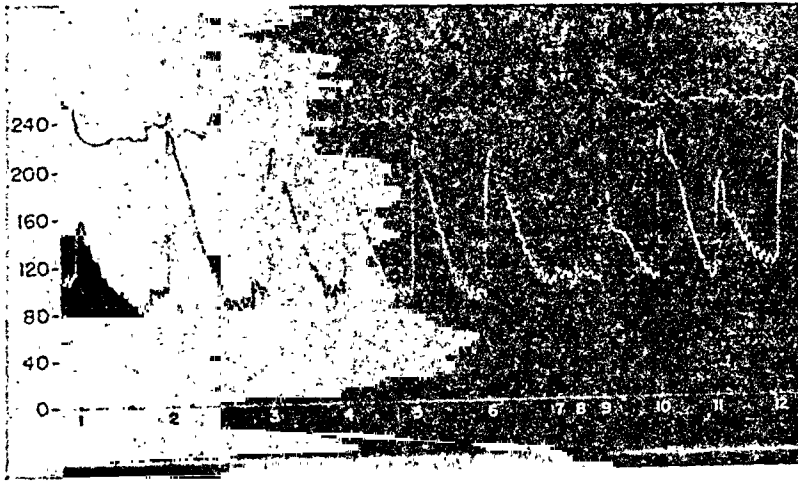


Fig. 3. EFFECT OF REPEATED DOSES of T.E.A. on anesthetized (pentobarbital) cat's blood pressure and nictitating membrane (sensitized by cocaine). Eight injections of 5 mg/kg. body weight T.E.A. had been given over a period of 5 hours before. (1) T.E.A. 5 mg/kg., (2) nor-adrenalin 0.015 mg. (3) T.E.A. 10 mg/kg., (4) adrenalin 0.0025 mg. (5) nor-adrenalin, (6) T.E.A. 10 mg/kg., (7) saline 3 cc., (8) cocaine 2 mg., (9) adrenalin, (10) nor-adrenalin, (11) T.E.A., (12) angiotonin 6 units.

equipressor amounts of L-nor-adrenalin than adrenalin. It must be recognized that we are presently uncertain of the L-nor-adrenalin content of commercial adrenalin.

That the relaxation is of neurogenic origin follows from the fact that none occurs when the membrane is denervated. Contraction results, we believe, because of a humoral agent. Further, this agent is blocked by Priscol just as is adrenalin and nor-adrenalin.

Three facts about the pressor response to T.E.A. stand out clearly: 1), that it is blocked when the response to nor-adrenalin is blocked, but does not parallel blocking of the adrenalin response: 2), that it does not occur in liverless animals except occasionally and in minor degree (i.e., 20 mm. Hg). This suggests that the pressor response is due chiefly to liberation of a nor-adrenalin-like substance from the liver. The adrenal glands seem to contribute to a far less important degree. 3) In the case of the animals with cord and sinus mechanism destroyed, the augmentation of the action of adrenalin and nor-adrenalin which we have described previously (3, 4) is an additional factor.

The vascular response to T.E.A. might be thought of as follows: At first, blockade

of autonomic ganglionic transmission causes loss of vasoconstrictor tone in the blood vessels comparable to that following nervous system destruction, and blood pressure falls. Administration of more T.E.A. produces progressively less depression until only pressor effects occur. Since the depressor action is greatly increased in liverless animals, it may be supposed that a buffer mechanism has been removed with excision of the liver. Since the pressor response to T.E.A. roughly parallels the response to nor-adrenalin and is concurrently blocked by Dibenamine, Priscol and Benzodioxane, and since little or no pressor responses occur in absence of the liver, the buffer mechanism must comprise chiefly liberation of a nor-adrenalin like substance from the liver. This substance may well be hepatic sympathin-N, following the nomenclature of v. Euler. A small component of the rise may be attributed to adrenalin because removal of both adrenal glands appears to reduce slightly the rise when the liver is intact, or abolish altogether the slight residual rise to be expected from T.E.A. sometimes seen after hepatectomy or blockade with Priscol and Benzodioxane. Since the sensitivity of the vascular tree is greatly heightened to both nor-adrenalin

TABLE 5. PRESSOR RESPONSE AND TETRAETHYLAMMONIUM CHLORIDE AUGMENTATION IN A HUMAN BEING SUFFERING FROM CEREBRAL THROMBOSIS

INITIAL BLOOD PRESSURE mm. Hg	TEST SUBSTANCE	TIME	BLOOD PRESSURE RISE mm. Hg
84	Adrenalin, 1 ml. 1:1,000	10:51	42
92	Barium chloride, 45 mg.	10:57	28
102	Angiotonin, 18 units	11:05	22
88	Tetraethylammonium, 700 mg.	11:15	8
94	Adrenalin, 1 ml.	11:18	38
100	Barium chloride	11:23	52
124	Adrenalin, 1 ml.	11:40	40
137	Tetraethylammonium, 600 mg.	11:42	31
120	Angiotonin, 18 units	11:46	50

and adrenalin by T.E.A., that liberated endogenously would be expected to elicit greater responses than in untreated animals.

Our experiments show that vasomotor impulses of the kind blocked by T.E.A. have their outflow from below D<sub>1</sub>; hence for routine studies, section of the cord at C<sub>6</sub> and destruction caudad is adequate. It was of especial interest that inactivation of the carotid sinus mechanism significantly heightened vascular responsiveness of the already highly sensitive animal. Administration of T.E.A. then caused insignificant further augmentation, although over a period of several hours the animals receiving T.E.A. were usually more sensitive than those not treated with it.

When the cord had been destroyed, the carotid sinus mechanism became much more conspicuous in its action. Its destruction in dogs with intact spinal cords produced little increase in responsiveness to vasoactive drugs, but with the cord destroyed heightening was pronounced. Further, during the early operations of carotid sinus destruction, most of our dogs died. When we realized that this was due to extreme vagus inhibition, and it was overcome, either by rapid cutting of the vagus nerves before sinus denervation, or by applying cocain solution to the sinus, there were no further deaths from the procedure. The unopposed carotid sinus mechanism when stimulated becomes a lethal one.

The ability of the vascular system to adjust to severe injury and the loss of organs doubtless contribute significantly to its regulation. This was shown, for example, by the experiments in which the spinal cord was destroyed 10 days before and the carotid sinus mechanism inactivated immediately prior to the experiment, and then the kidneys, the adrenal glands and liver excised under ether anesthesia. These were not moribund animals, nor was anesthesia required after operation. Eight hours after such extensive surgery, the mercury manometer recording of blood pressure was indistinguishable from that of normal dogs. This is not to imply that the responses were the same. Rather, to be emphasized is the extraordinary power of the circulation to perform its primitive functions temporarily, at least, in the absence of vital organs. Reinhard, Glasser and Page (14) have shown that hepatectomized dogs with or without their kidneys withstand hypotension of long duration quite as well as more normal animals. It would seem unreasonable to suppose, however, that the vascular responses are identical.

Finally, the elucidation of the mechanism of T.E.A. action shows why the drug has not proved a satisfactory indicator of sympathetic activity. For instance, Birchall, *et al.* (6) found no correlation between the response to surgical sympathectomy in hypertensive patients and the chemical sympathectomy performed by T.E.A. If the response to T.E.A. has two components, the one depressing blood pressure the other elevating it, the resultant will always be a compromise between the magnitude of these forces. When most of the pressor factor is removed by hepatectomy-adrenalectomy, then a quarter of the usual dose produces severe unopposed fall in blood pressure. If the nervous factor (autonomic blockade) is abolished by previous destruction of the spinal cord, only a rise occurs. Thus, to determine the influence of the drug on ganglionic blockade alone on blood pressure, it is necessary to exclude an augmented humoral factor, having opposing effects to those of blockade.

Bilateral nephrectomy performed two days before the test is an example of a means of affecting the normal equilibrium between the two opposing factors. A common response in such animals to the initial T.E.A. dose is a rise in arterial pressure with little or no fall, as opposed to the usual fall in normal dogs. It might therefore be supposed that nephrectomy causes the retention of substances acting to reduce the vasomotor factor, leaving the humoral mechanism unopposed.

It should be noted that we were unable to detect any consistent change in the response to T.E.A. after renal hypertension was produced by wrapping the kidneys in silk. The fall of blood pressure in mm. Hg was roughly the same before and after hypertension. This is consistent with the demonstration by Freeman and Page (15) that the autonomic system does not exert a dominant influence in this particular type of hypertension.

#### SUMMARY

The blood pressure response of a large number of anesthetized dogs to intravenous injection of 5 or 10 mg/kg. body weight of tetraethylammonium chloride (T.E.A.) was irregular, ranging all the way from pure depressor to pressor, varying even from day to day in the same animal.

There was no consistent change in the response after the development of ex-

perimental renal hypertension by silk perinephritis. A spontaneous type of vascular refractoriness to a variety of vasoactive substances not infrequently occurred, often but not necessarily associated with a period of hypotension. Anterior rhizotomy from C<sub>6</sub> to L<sub>4</sub>, 'total' surgical lumbodorsal sympathectomy, cord destruction from C<sub>6</sub> caudad or removal of section of cord between C<sub>5</sub> and C<sub>6</sub>, all abolished the depressor component of T.E.A., leaving a pure pressor response. Inactivation of the carotid sinus mechanism in otherwise normal dogs caused little change in response but in dogs with the cord destroyed from C<sub>6</sub> caudad, the sinus mechanism was highly active. Its inactivation in cord-dogs augmented the pressor response elicited by T.E.A.

Bilateral nephrectomy increased the tendency for a pressor response to occur at the expense of the depressor. Bilateral adrenalectomy accentuated the depressor responses at the expense of the pressor. Hepatectomy greatly augmented depressor responses and all but abolished the pressor ones. If adrenalectomy was combined with hepatectomy and nephrectomy, no pressor response was observed. Removal of the liver and kidneys in animals with the cord destroyed and the sinus mechanism inactivated abolished the pressor responses to T.E.A. Administration of doses of Priscol, Dibenamine and Benzodioxane, singly or combined, sufficient to reduce or block the response of arterenol also reduced or blocked the pressor response of T.E.A. Cats exhibited the same reversal from depressor to pressor response after injection of T.E.A. Relaxation of the nictitating membrane occurred regularly until well after the pressor response was established, then quickly reversed to contraction. The degree of contraction followed much more closely that due to equipressor amounts of nor-adrenalin than adrenalin or angiotonin. No relaxation occurred after the membrane was denervated but contraction was observed when the depressor action of T.E.A. was over. Injection of Priscol then abolished the contraction of the membrane to adrenalin, nor-adrenalin and T.E.A. Two patients with injury to the central nervous system have shown the initial pressor response to T.E.A. which occurs in dogs with parts of the nervous system removed.

#### CONCLUSION

These observations in dogs, cats and man suggest that the action of tetraethylammonium chloride (T.E.A.) on arterial pressure is compounded of at least three factors: 1) autonomic blockade reducing or eliminating tonic vasomotor impulses which lowers arterial pressure, 2) stimulation of the liver, to liberate a nor-adrenalin-like substance which, acting on a more than normally sensitive vascular tree, raises blood pressure, 3) less importantly, liberation of adrenalin from the adrenal glands. The net effect on arterial pressure is the resultant of the forces tending to lower opposed by those tending to elevate it.

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# CARDIOVASCULAR EFFECTS OF LARGE VOLUMES OF ISOTONIC SALINE INFUSED INTRAVENOUSLY INTO DOGS FOLLOWING SEVERE HEMORRHAGE<sup>1,2</sup>

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THE use of normal salt solution to restore a large blood deficit caused by hemorrhage has had few supporters. Harkins and McClure (1) in a review of the literature up to 1941 mentioned many investigations indicating that isotonic saline was the least beneficial of replacement fluids. In general, the arguments raised against saline were: *a*) that an isotonic crystalloid solution, having no colloid osmotic pressure, would be lost from the circulation too rapidly to exert more than temporary benefit, *b*) that loss into the tissue might even cause pulmonary edema (2), and *c*) that, where capillary permeability was increased, it might wash plasma proteins out of the blood (3) leaving the circulation in a worse state than before the infusion of the salt solution. There were, however, some supporters for the use of saline, namely Hoitink (4) and MacFee and Baldridge (5), the latter having success with the therapeutic use of large volumes of the fluid.

In March, 1942, the first of a series of articles by Rosenthal (6) appeared. Investigating the most effective treatment for burns and shock, produced in large numbers of mice, he found that oral, intravenous or intraperitoneal administration of isotonic (0.9 per cent) NaCl solution significantly reduced the mortality rate. Further experiments by Rosenthal (7-10) on tourniquet shock and hemorrhage indicated that transfusions with large volumes (8-15 per cent of the body weight) of isotonic saline were as effective in reducing mortality as transfusions with serum or whole blood. Fox (11) who tried large amounts of sodium lactate solution orally in severely burned patients reported favorable results. Sodium chloride solution is efficacious in preventing shock produced in dogs by venous occlusion according to Katz, Friedberg and Asher (12) who concluded from their experiments that the beneficial effect can be attributed to the sodium ion. Warren, Merrill and Stead (13), who studied the effects of saline infusion on tourniquet shock in dogs, felt that the hydration of the interstitial fluid compartment was the important factor in the maintenance of the blood volume. After removing the tourniquets they gave saline intravenously in large amounts and over long periods of time, and they reported that the plasma volume and arterial pressure were maintained at normal levels when the infusion was carried to the point of development of generalized edema. The fact is that the investigations in which isotonic saline was found to be beneficial are those in which the volumes given amount to two or more times the estimated blood volumes.

The purpose of the experiments reported in this paper has been to analyze the circulatory changes brought about by the infusion of large volumes of saline after severe hemorrhage and to ascertain to what extent the circulatory changes themselves may account for the favorable effects.

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## PROCEDURE

Adult, mongrel dogs ranging in weight from 5 to 10 kg. were used. Each animal was offered unlimited water but no food for 24 hours before the start of the experiment. The dog was placed on his back on an animal board. The right and left femoral arteries and the right jugular vein were exposed under local procaine hydrochloride supported by Neothlesol.<sup>3</sup>

The experiment consisted of a hemorrhage followed an hour later by a large intravenous infusion of isotonic saline. The clinical condition of the animal and especially the cardiovascular responses were observed for 24 hours after the bleeding. A dog living that long was considered a survivor. During the control period values for the heart rate, mean arterial pressure (arterial puncture, Hg manometer) and O<sub>2</sub> consumption were obtained, and blood samples were drawn for the estimation of plasma volume, plasma protein concentration, hematocrit readings and arterial and mixed venous O<sub>2</sub> content. The rectal temperature was taken only during the control period. Any dog showing evidence of fever was not used.

The hemorrhage was performed as described by Walcott (14) except that during the period following bleeding only one 7 cc. sample of blood was withdrawn. All of the above measurements, with the exception of the plasma volume, were repeated during the interval between bleeding and infusion as well as during the period following saline infusion. The saline used was a 0.9 per cent solution of chemically pure sodium chloride and distilled water freshly prepared for each experiment. It was warmed to 39° C. and injected through a catheter inserted in the right jugular vein. A rate of about 1.3 cc/min/kg. was empirically chosen from preliminary experiments (15). The administration of saline was stopped when the dog had received a volume equal to 15 per cent of the body weight. When the measurements were completed, one and a half to two hours later, the animal was removed from the board and placed in a metabolism cage. Water was given *ad libitum* but no food, and the measurements were repeated the following morning on all survivors.

Each dog was weighed before the experiment, at the end of the first day and before being put on the board the second morning. In most of the animals the bladder was emptied and washed out through a catheter immediately before starting the infusion; urine was collected during and following the administration of saline. The overnight urine loss as well as the amount of water drunk during the night were measured roughly on all except a few dogs.

The plasma volume was determined, using the dye T-1824 and the method described by Gregersen and Stewart (16). The dye was injected into the right jugular vein. For the control volume samples were drawn from the left jugular vein, while those for volumes measured on the following day were drawn from a femoral artery. The optical densities of the dye samples were determined with a König-Martens spectrophotometer (17). Semilog plots of the optical densities were used for calculating plasma volumes (18). Hematocrit values were obtained by centrifuging heparinized blood samples in Wintrobe tubes for 30 minutes at 3000 r.p.m. (radius 13 cm.). The total blood volume was calculated in the usual way (18a), using 0.96 as the correc-

<sup>3</sup> Two parts of methyl methylene para amino phenylformate and 5 parts of hydroxybenzocresol in refined almond oil. Caso Laboratories, New York City.

tion factor for the plasma trapped among the erythrocytes (19). The plasma protein concentration of each sample was measured with an Abbé refractometer (20).

The direct Fick method was used for determining the cardiac output. Arterial blood samples were drawn from a femoral artery into syringes rinsed with heparin solution. To obtain mixed venous blood a catheter, attached to a saline manometer, was inserted through the left jugular vein and manipulated into a position which, according to the criteria used, was considered to be in the right ventricle. The depth of the catheter, the characteristic impact of systole felt and seen along the tube and the manometric readings were the criteria used in judging the position of the catheter. For the determinations made during the control period and after the infusion the pressure readings were always between 20 and 10 cm. saline, and the systolic impact was firm and forceful. For the post-hemorrhagic determinations, however, the impact was feeble and the pressure was about zero. On the second day of the experiment the catheter's position was confirmed at autopsy and, in all except 3 animals, the tip of the catheter was found in the right ventricle. In one of these the tip of the tube was in the vena cava at the entrance to the auricle, and in the other two it was in the pulmonary artery. In order to avoid the presence of saline from the manometer in the blood samples the catheter was first flushed out by withdrawing 10 cc. into a separate syringe. After obtaining the mixed venous sample the 10 cc. of saline and blood were returned. Throughout the experiment the dripping of saline from the manometer's reservoir into the vascular system was prevented except during the control period when not more than 3-cc. passed through the catheter. Clamping off the catheter from the manometer after the former was filled with saline stopped the flow. This was sufficient to prevent clotting in the tube.

Arterial and mixed venous blood samples were analyzed for their respective  $O_2$  contents by the Roughton and Scholander method (21). Analyses were made from 6 to 8 hours after obtaining the blood, the samples being stored as described by Roughton and Scholander. In agreement with these investigators, the precision of measuring  $O_2$  content on the same blood sample was found to be  $\pm 0.2$  vol. per cent.

Oxygen consumption was measured by means of a Benedict-Roth spirometer. The dog breathed through a Blalock mask. Great care was taken in the application of the mask and in holding the dog's head during recording periods in order to avoid a leak around the muzzle. The concentration of  $O_2$  within the spirometer was maintained at  $35 \pm 5$  per cent by filling the gas chamber about one seventh with pure  $O_2$ , the remainder being room air. Three minutes after starting to record  $O_2$  consumption the first venous sample was drawn, followed 20 seconds later by an arterial blood sample. Without changing the arrangement in any way, another pair of samples was drawn two minutes later. The  $O_2$  contents of the two arterial samples were averaged, as were the two venous  $O_2$  contents, and the difference between the averages used in calculating the cardiac output.

## RESULTS

*Changes in Fluid Volumes, Hematocrit Values and Plasma Protein Concentration.* Of the 27 dogs studied, 23 survived. The second day of each experiment the survivors were alert, responsive and walked and trotted when led from their cages.

Figure 1 shows the mean values of most of the fluid exchange data which were obtained from the 23 survivors. The average weight was 7.75 kg., the range extending from 4.8 to 11.3 kg. Urine outputs and water intakes though measured were not averaged because the range of variation was too great for the values to be significant. In spite of removing an average volume of 326 cc. of blood (42 cc/kg.) during the hemorrhage and of injecting an hour later an average of 1170 cc. of saline (151 cc/kg.), the mean weight increase at the end of the first day was only 0.25 kg. All dogs which

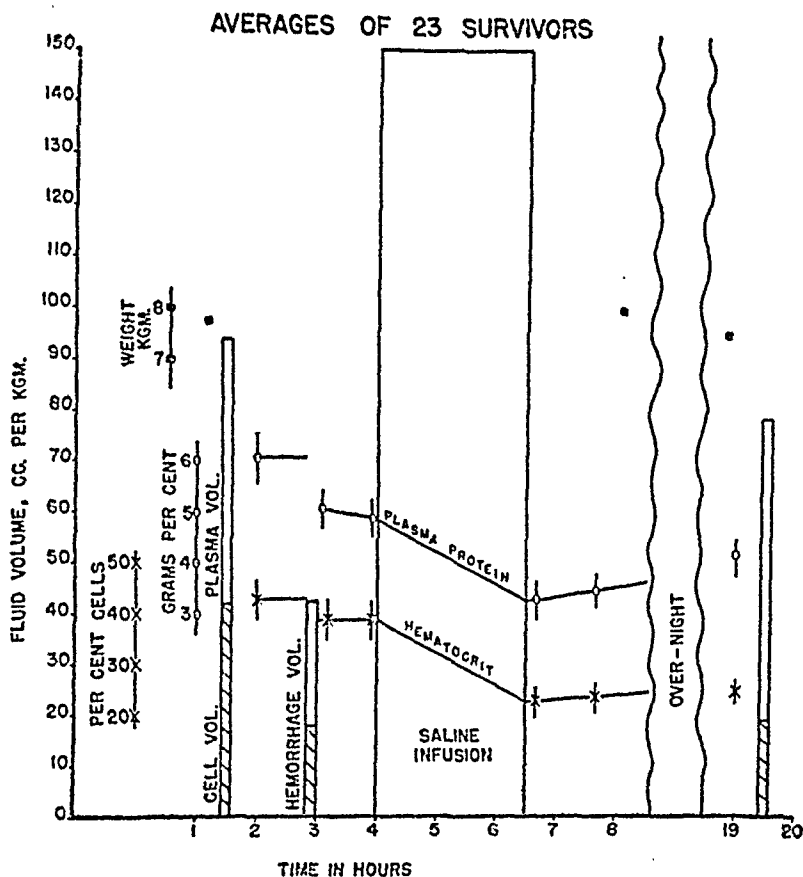


Fig. 1. EFFECT OF HEMORRHAGE and saline infusion on body weight, plasma volume, plasma protein concentration and hematocrit value in experiments on 23 surviving dogs. Average values are indicated by the characteristic points and average deviation is shown by the vertical line drawn through the point. Time values also represent averages.

survived showed a considerable diuresis which began within an hour after the start of the infusion. The rate of outflow increased until in some experiments more than 250 cc. was collected in the third hour. Even taking into account the urine output, the weight at the end of the day is lower than might be expected. The unmeasured loss of water from the lungs and skin would account for this. No diarrhea or vomiting was shown during the day by any of the dogs which survived. The diuresis continued throughout the night. When placed in his cage at the end of the first day each dog immediately drank several hundred cc. of water. During the night much more was ingested. Although the volume of fluid exchanged overnight varied considerably in different animals, the weight the next morning consistently showed a loss which averaged 0.2 kg. less than the average control weight.

The plasma and blood volumes, measured one hour before the hemorrhage, averaged  $52 \pm 4$  cc/kg. and  $94 \pm 7$  cc/kg., respectively. On the day after the infusion the plasma volume of every survivor, with the exception of one dog, showed an increase, the mean value reaching  $60 \pm 4$  cc/kg. The red cell volume, however, was so greatly reduced by the bleeding that the average total blood volume measured the next day was only  $79 \pm 6$  cc/kg.

The plasma protein concentration fell from an average control value of  $6.1 \pm 0.5$  gm. per cent to  $4.9 \pm 0.4$  gm. per cent one hour after the hemorrhage and after the saline infusion it was further reduced to  $3.3 \pm 0.4$  gm. per cent. One would expect both of these changes, the first resulting from the compensatory dilution of the blood by the tissue fluids during and after hemorrhage, and the second resulting from the dilution of plasma by the saline infusion. The mean hematocrit values which are also listed in table 1 show the same relative changes. On the following day, however, the plasma protein concentration was found to have increased significantly but the hematocrit value remained about the same.

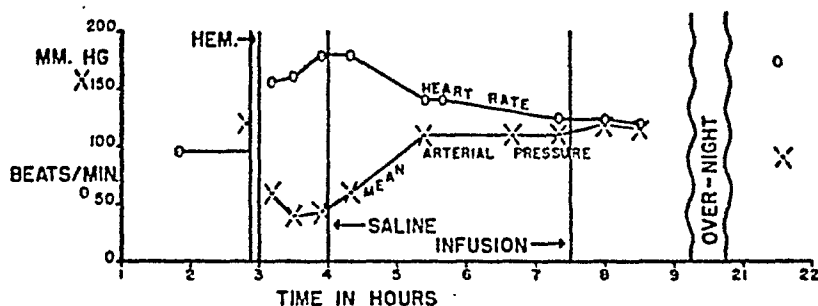


Fig. 2. HEART RATE AND BLOOD PRESSURE changes during a typical experiment; values of a single dog, no. 4.

*Circulatory and  $O_2$  Consumption Changes.* The two curves in figure 2, representing typical fluctuations in blood pressure and heart rate, were taken from the protocol of experiment 4. After the hemorrhage, the mean arterial pressure fell from a control value of 120 mm. Hg to 60 mm. Hg. In many instances the fall was greater. In this dog the pressure remained low, 40 to 50 mm. Hg, until the infusion started, whereupon it rose rapidly. By the end of the infusion the value was about 115 mm. Hg where it remained during the rest of the first day. The heart rate invariably rose after the hemorrhage; in the animal shown in figure 2 it rose from 96 to 156 beats per minute. It continued to increase to 180 until the infusion was given, after the start of which it decreased, leveling off first at 140 and later at 120. On the following day the heart rate was 170, and the blood pressure was 90 mm. Hg. The variations in blood pressure and pulse of all the survivors were of this order, and especially consistent were the second day's findings that the heart rate was 70 to 100 beats per minute above and the blood pressure 20 to 30 mm. Hg below the control value.

Mean values and standard deviations for other circulatory changes and for  $O_2$  consumptions are shown in table 1. The latter when measured about 15 minutes after hemorrhage was found to have decreased an average of 26 per cent. Thereafter, it began to recover in 14 animals but remained the same or fell still lower in 9 dogs. The determinations which were made directly after saline infusion showed an increase

in 13 animals. By calculating the  $O_2$  consumption on the basis of cc/min/kg., the range of variability was reduced from a standard deviation of about 5 per cent to one of about 3 per cent. Little improvement was shown in the metabolic rate according to the final determinations made on the first day, but on the second day the  $O_2$  consumption of all except 5 dogs exceeded the control levels. The control  $O_2$  consumption values for 4 of the 5 dogs were unusually high. Since the heart rates of these animals were also high, there seems little question that the animals were excited during the control period.

The hemorrhage did not greatly alter the arterial  $O_2$  content, but, as expected, the venous  $O_2$  content was greatly reduced. Thus, an average A-V  $O_2$  difference of

TABLE 1. EFFECTS OF HEMORRHAGE AND SALINE INFUSION ON  $O_2$  CONSUMPTION, HEMATOCRIT VALUE, ARTERIAL AND VENOUS  $O_2$  CONTENT, CARDIAC OUTPUT AND PERIPHERAL RESISTANCES

	BEFORE HEMORRHAGE	$\frac{1}{2}$ HR. AFTER HEMORRHAGE	1 HR. AFTER HEMORRHAGE	$\frac{1}{2}$ HR. AFTER INFUSION	1 $\frac{1}{2}$ HR. AFTER INFUSION	19 $\frac{1}{2}$ HR. AFTER INFUSION
$O_2$ consumed cc/min/kg.	11.1 $\pm$ 1.5	8.2 $\pm$ 1.4	9.3 $\pm$ 1.2	9.9 $\pm$ 1.4	9.8 $\pm$ 1.0	11.1 $\pm$ 1.6
Hematocrit % R.B.C.	43.2 $\pm$ 4.1	38.9 $\pm$ 3.9	38.9 $\pm$ 3.5	22.5 $\pm$ 2.8	24.3 $\pm$ 3.0	25.2 $\pm$ 2.4
Arterial $O_2$ vol. %	18.1 $\pm$ 1.6	16.0 $\pm$ 1.7	16.4 $\pm$ 1.5	9.2 $\pm$ 1.0	9.7 $\pm$ 1.1	9.9 $\pm$ 1.1
Mixed venous $O_2$ vol. %	13.8 $\pm$ 1.8	4.0 $\pm$ 1.8	4.9 $\pm$ 2.2	5.2 $\pm$ 1.4	5.3 $\pm$ 1.1	5.7 $\pm$ 1.3
Cardiac out- put cc/min/kg.	274 $\pm$ 54	74 $\pm$ 22	86 $\pm$ 21	265 $\pm$ 58	229 $\pm$ 44	274 $\pm$ 65
Peripheral re- sistance A.U.	5400 $\pm$ 1500	5900 $\pm$ 1600	4900 $\pm$ 1100	5100 $\pm$ 1300	5400 $\pm$ 1100	3800 $\pm$ 110

The quantities represent mean value with average deviations from the mean and were obtained from measurements on 23 surviving dogs. Time values are also averages.

$$\text{Peripheral resistance} = \frac{\text{mean arterial pressure}}{\text{cardiac output/sec.}} \times 1332 \text{ in } \frac{\text{dynes second}}{\text{cm.}^5} \text{ absolute Units.}$$

A. U. = Absolute Units.

12.0 vols. per cent was observed in contrast to the average control difference of 4.3 vols. per cent. After the infusion the arterial  $O_2$  content was decreased an average of 7.2 vols. per cent by the dilution of the blood. Since changes in arterial  $O_2$  followed closely the changes in the concentration of red cells, it may be inferred that the percentage saturation of the arterial blood remained relatively unchanged. The average venous  $O_2$  content increased only slightly before the start of the infusion, from 4.0 to 4.9 vols. per cent, and continued near this latter value throughout the rest of the day. The cardiac output, reduced to 27 per cent of the control value by the bleeding, showed a slight improvement in 14 of the animals during the hour following hemorrhage. After saline infusion, however, the increase above pre-infusion levels was striking. In 11 dogs the cardiac outputs were higher than the control value, the

average for all 23 dogs being only 4 per cent below the average control value of 274 cc/min/kg. Thus, with the lowered arterial O<sub>2</sub> concentration and with the venous O<sub>2</sub> concentration maintained by the improved outflow of blood from the heart, the A-V difference was decreased to the control range. This favorable condition continued not only for one hour after the infusion but was found to be about the same the following morning, some 19 to 21 hours after blood loss. At this time the average A-V difference was still approximately 4.4 vols. per cent, and the mean value for the cardiac output was the same as the mean control value, 274 cc/min/kg.

Peripheral resistance was calculated according to the method described by Wiggers (22) and the values obtained are included in table 2. There was a wide variation of response as shown by the large standard deviations. In most of the dogs, however, the resistance increased after the bleeding. The average control value for all survivors was 5400 A.U., which increased to a post-hemorrhage value of 5900 A.U. During the hour before the saline there was a decline to an average of 4900 A.U. The measurements following the infusion showed the changes to be highly individual, the mean value being 5100 A.U. immediately afterwards and 5400 A.U. one hour later.

TABLE 2. MEAN VALUES AND AVERAGE DEVIATIONS FROM THE MEAN OF TOTAL AMOUNTS OF CIRCULATING PLASMA PROTEIN

NO. OF SURVIV- ING DOGS	T.P. (a)	T.P. (b)	T.P. (c)	INCREASE IN T.P.	
	gms.	gms.	gms.	gms.	gm./kg.
23	24.8 ± 4.6	11.4 ± 2.6	18.7 ± 3.8	7.2 ± 2.4	0.9 ± 0.2

T.P. (a) = Total plasma protein calculated from plasma volume and plasma protein concentration measured during the control period. T.P. (b) = Total plasma protein lost during the hemorrhage. T.P. (c) = Total plasma protein calculated from measurements made on the following morning.

On the following day every dog, with one exception, showed a decrease in peripheral resistance. The average value at that time was 3800 A.U.

In preliminary experiments (15), 2 dogs, which were given saline equal to 18 per cent of the body weight, developed pulmonary edema. However, in the experiments reported in which saline amounting to 15 per cent of the body weight was administered the only marked edema seen was in the paws and around the eyes. These signs had disappeared by the following morning.

The various determinations made on the 4 non-survivors showed values not strikingly different from the averages for the survivors. No common denominator could be found which would account for all four deaths. Dog 25 had a blood pressure of 100 mm. Hg and a heart rate of 110 beats per minute at the end of the first day. He died about 21 hours after the hemorrhage. Autopsy revealed no cause. Dogs 24 and 26 rallied only temporarily from the post-hemorrhagic hypotension. Their mean pressures rose to 90 and 60 mm. Hg respectively, but half way through the saline injection the pressures began to fall. When put in their cages the pressures were 45 and 18 mm. Hg respectively. No urine could be collected from the bladder; instead profuse diarrhea appeared. The same response was shown by dog 27 except that the mean arterial pressure was better maintained and some urine was formed. In this instance fluid was lost by vomiting as well as by diarrhea.

## DISCUSSION

Since 23 of the 27 dogs survived it is of interest to consider how many might have lived if no saline infusion had been given after the hemorrhage. It has been found by Wang *et al.* (23) that 50 per cent survival (L.H.<sub>50</sub>) can be expected if there is a residual blood volume of 59 cc/kg. one hour after the hemorrhage. In the experiments reported in this paper the blood volume was not measured after the bleeding. Therefore the post-hemorrhagic residual blood volume can only be roughly estimated. With an average control blood volume of 94 cc/kg. and an average hemorrhage volume of 55 cc/kg., an uncompensated residual blood volume of 39 cc/kg. would have been expected. However, during the bleeding, tissue fluid enters the blood vessels, diluting the serum proteins. Walcott (24) found that in 23 dogs the compensatory reserves involved in the early restoration of blood volume ranged from 3 to 18 per cent of the control blood volume, the average being 10.7 per cent. This mean value has been used in Walcott's formula to estimate the residual blood volume at the time of the start of the saline infusion for each dog used in these experiments:  $0.107 \text{ B.V.} + (\text{B.V.} - \text{H.V.})$  in which B.V. represents control blood volume and H.V., hemorrhage volume. The average calculated residual blood volume for all of the dogs was 61 cc/kg. for which, according to Wang's figures, a 58 per cent survival might be expected. Even though this estimated residual blood volume is a rough approximation the survival of 23 out of 27 dogs indicates that the infusions were of therapeutic value.

Direct evidence of the beneficial effect of saline on the survival rate of hemorrhage dogs was obtained from experiments on 6 animals (25) which were subjected to a Walcott hemorrhage (14). Except for the fact that no saline infusions were given to these 6 dogs the experimental conditions were the same as for the 27 dogs whose data are being reported in this paper. Only one dog was alive 24 hours after the bleeding. The other 5 died within 2 to 5 hours after the hemorrhage.

Approximately 20 hours after the end of the saline infusion the plasma volume averaged 8 cc/kg. greater than before the hemorrhage. Plasma volume changes before, during and after the infusion can only be inferred from changes in plasma protein concentration and hematocrit values. The similarity in the changes of these two throughout the first day of the experiment show that the plasma compartment increased during the infusion and declined slightly when the inflow was stopped.

On the following day the average plasma protein concentration had increased significantly more than had the average percentage of red cell volume. From the measurements on each dog calculations were made of the total amount of plasma protein in a) the control plasma volume, b) the plasma lost during hemorrhage, and c) the post-infusion plasma volume. Averages of these calculations appear in table 2. When allowance is made for the amount of protein lost during hemorrhage, the total plasma protein found on the second day was in each instance larger than might have been expected. The average increase was 0.9 gm/kg. These results show that the significant increase of the plasma protein concentration on the day after hemorrhage was caused partly by the addition of protein to the circulation rather than entirely by a loss of plasma. The experiments of Beard *et al.* (26) and those of Stewart and Rourke (27) showed similar results when normal salt solution was injected intravenously following a hemorrhage. Ebert *et al.* (28) found that the plasma proteins increased after hemorrhage even though no transfusion was given.

From the average hematocrit values in figure 1 it can be seen that on the day after bleeding the percentage of red cells was approximately equal to the value found after infusion. This does not necessarily mean that the plasma volume remained unchanged, for according to Huber (29) the total red cell volume decreased 24 hours after hemorrhage. Probably a similar change occurred in these experiments.

Objection to the use of saline as a blood substitute has been raised on the grounds that it is too easily lost from the circulatory system, thus yielding only a temporary increase in blood volume. As is well known sodium and chloride ions and water are freely diffusible through capillary walls, and so, limited only by the diffusion gradients of the ions, saline can move quickly into the tissue spaces which are dehydrated by exsanguination. Stewart and Rourke (27), who gave humans large isotonic saline injections over a period of 8 days, found that the salt solution was held in the extracellular fluid compartment. They did not measure how long saline is retained after the end of the infusion.

In the present experiments saline equal to almost twice the control blood volume was infused. The mean arterial pressure never rose above 125 mm. Hg. Much of the injected solution probably entered the interstitial spaces and remained there for at least a few hours. This is confirmed by the observed increase in the weight of the animals and the presence of cutaneous edema. Twenty hours later, however, the extensive diuresis, the weight loss and the disappearance of the edema indicate a general fluid loss, which is probably extracellular. The success with large volumes of isotonic fluid has been explained by Warren, Merrill and Stead (13) who suggest that when enough saline fills the interstitial compartment, the tissue pressure is raised high enough to oppose the capillary hydrostatic pressure. This, they believe, helps to retain saline in the vascular bed so that the blood volume cannot only be restored but also can be maintained at a normal level. In the experiments reported in this paper it appears that though a similar equilibrium between hydrostatic and tissue pressure may have been reached toward the end of the saline infusion, most of the infused solution had been lost by the next day.

The events which occur in the exsanguinated dog result from the actual loss of blood volume. Alterations in the cardiac output (30, 31),  $O_2$  consumption, venous  $O_2$  content and other chemical (32), as well as clinical (18a), observations indicate a reduced blood flow through the tissues, thus bringing about the development of stagnant anoxia (30). The data reported in this paper indicate that isotonic saline administered in sufficiently large amounts restores the total blood flow, for after the infusion the heart, on the average, expelled as much blood as it had before the hemorrhage. To be sure, the  $O_2$  content of arterial and venous blood remained low, but the significant point is that the A-V  $O_2$  difference was restored to the normal value. The venous  $O_2$  content is maintained by the improved blood flow rather than by lack of  $O_2$  utilization. There was close agreement in all arterial  $O_2$  measurements between the hematocrit value and the  $O_2$  content. Thus, at all times the blood received by the tissue cells was of a high percentage saturation. In venous blood, however, the  $O_2$  tension fell to low values after the hemorrhage but was restored by the increased cardiac output to normal limits following the infusion. The next morning this improved cardio-vascular condition was still present. The cardiac output and the

venous  $O_2$  content had increased slightly. Oxygen consumption was restored to the control rate. In spite of the fact that the total blood volume was still 16 per cent below the control measurement, considerable circulatory recovery had taken place.

In the experiments reported here not only was the proportion of surviving dogs high but not one of the dogs which lived developed any of the clinical symptoms of shock, such as apathy, pale, dry mucous membranes of the mouth, cold limbs or collapsed veins (33). The fact that the saline infusion was started as early as one hour after the severe hemorrhage may have prevented the development of the metabolic changes which lead to a condition of shock. By increasing the total systemic blood flow during the hours just after hemorrhage the saline administration may have helped the animal through a critical period until its own defense mechanisms, such as addition of protein to the blood, could be brought into play. On the following day, though little of the infusion fluid remained, the cardiac output was sufficiently restored so that with adequate care and feeding each dog probably could be returned to a healthy, normal condition in time.

#### SUMMARY

A study has been made of the effects of hemorrhage and of an intravenous infusion of a large volume of isotonic salt solution on the circulatory system. Calculated residual blood volumes average 61 cc/kg. one hour after hemorrhage. Nevertheless, 23 of the 27 dogs survived. In six control experiments 5 dogs died within 2 to 5 hours after the hemorrhage.

The average hematocrit reading was decreased by the saline infusion. Eighteen hours later it had not varied significantly from this post-infusion level. Average values for plasma protein concentration showed a similar decrease following the saline infusion but had increased significantly the following day (fig. 1). Calculation of the total circulating plasma protein showed that after hemorrhage an average of 0.9 gm/kg. of body weight had been added to the blood. After infusion the plasma volume averaged 8 cc/kg. above the control value, but the total blood volume was 15 cc/kg. below the control value. The dogs drank copiously and excreted large volumes of urine.

The average cardiovascular changes can be seen in table 1. Oxygen consumption showed an average reduction of 27 per cent following hemorrhage but after the infusion it was only 10 per cent less than the control values. By the next day the average  $O_2$  consumption was the same as the control rate. The average control cardiac output of  $274 \pm 54$  cc/min/kg. was reduced to one fourth immediately following hemorrhage. The saline inflow brought it up to  $265 \pm 58$  cc/min/kg., and the following day it was  $274 \pm 65$ , the same as the control output. In accord with the hematocrit changes, the average arterial  $O_2$  content fell after the infusion from 16.4 to 9.2 vols. per cent, being slightly increased on the second day. The venous  $O_2$  content, though lowered from 13.7 to 4.0 vols. per cent by the bleeding, rose after the infusion to 5.2 vols. per cent and remained in that range. Thus, the average A-V  $O_2$  difference increased from a control of 4.3 vols. per cent to one of 12.0 vols. per cent after hemorrhage. The infusion restored it to 4.0 vols. per cent and it was not significantly changed 24 hours later. These circulatory changes induced by the large saline infusion offer an explanation of the beneficial effects observed.

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# EFFECTS OF CHANGES IN BODY TEMPERATURE AND INSPIRED AIR HUMIDITY ON LUNG EDEMA AND HEMORRHAGE<sup>1</sup>

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USING the resistance breathing method described by Zinberg, Nudell, Kubicek and Visscher (1), production of pulmonary edema appeared to be increased when dogs spontaneously developed hyperthermia on warm humid days (2). On cooler days, when hyperthermia was intentionally produced by having the animals breathe 100 per cent humidified air warmed to 37° C., severe pulmonary edema again developed. In order to determine whether the warm humidified air or hyperthermia was the causative factor, it was decided to use guinea pigs where variables could be more easily controlled. Sussman, Hemingway and Visscher (3) have shown that guinea pigs subjected to artificial respiration of 20 mm. Hg positive pressure insufflation by tracheal cannula for 3 to 6 hours regularly develop pulmonary edema. Using shorter time periods and slightly lower pressures to create minimal edema, the animals reported in the paper were studied in regard to environmental, body and airway temperature in addition to the humidity of the air breathed. Hall and Wakefield (4), Jacobsen and Kiyoshi (5) and others mentioned pulmonary edema, hemorrhage and congestion among the pathological findings at autopsy in a small percentage of animals subjected to hyperthermia. Malamud, Haymaker and Custer (6), in a clinico-pathological study of 125 human victims of heat stroke, reported elevated lung weight due to hemorrhage, edema and congestion in all cases. Cardiac hemorrhages were also frequently present, confirming previous findings of Wilson (7).

## METHODS

Adult guinea pigs of both sexes weighing between 228 and 729 gm. were employed. They were anesthetized with intraperitoneal sodium pentobarbital, 3.0 mg/100 gm. Artificial respiration by positive pressure tracheal cannula insufflation was maintained throughout. The maximum pressure of insufflation was 18 mm. Hg. Expiration was passive, at atmospheric pressure. Two animals, closely paired for weight, were studied simultaneously on the same respiration pump. One animal acted as a control breathing room air while the second animal, in Series I of the experiment, was required to breathe 100 per cent humidified air warmed to its body temperature from a humidifier interposed in the respirator system. In Series II, the second animal was placed in a warming box to produce hyperthermia while breathing the same room air as its control. In Series III, the second animal breathed warm humidified air in addition to being subjected to the elevated environmental temperature of the warm-

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TABLE I

Series I						Series II						Series III					
CONTROL			HUMIDIFIED AIR AT BODY TEMPERATURE			CONTROL			HYPERTHERMIA			CONTROL			HYPERTHERMIA PLUS HUMIDIFIED AIR AT BODY TEMPERATURE		
$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	$\frac{L}{V}$	Max. body temp. °C.	Notes	$\frac{L}{B} \times 10^4$	Max. body temp. °C.
85 <sup>1</sup>	3.26	35.6	S <sub>3</sub>	116 <sup>2</sup>	4.28	38.8	S <sub>3</sub>	76	2.85	35.8	S <sub>1</sub> <sup>†</sup>	93 <sup>2</sup>	3.88	43.6	S <sub>1</sub> <sup>†</sup>	98	3.20
86	3.20	36.8	S <sub>3</sub>	111 <sup>1</sup>	4.01	37.7	S <sub>3</sub>	77	2.82	35.4	S <sub>2</sub>	66 <sup>2</sup>	2.83	40.2	S <sub>1</sub> <sup>†</sup>	108	3.81
163 <sup>1</sup>	6.33	34.8	S <sub>3</sub>	127 <sup>1</sup>	4.98	34.7	S <sub>3</sub>	116 <sup>2</sup>	4.46	35.2	S <sub>2</sub>	85 <sup>2</sup>	3.48	44.9	S <sub>2</sub> <sup>†</sup>	67	2.88
109 <sup>1</sup>	4.10	36.7	S <sub>3</sub> <sup>†</sup>	91 <sup>1</sup>	3.35	36.3	S <sub>3</sub>	79	3.40	38.2	S <sub>1</sub> <sup>†</sup>	184 <sup>2</sup>	7.15	44.4	S <sub>1</sub> <sup>†</sup>	85 <sup>1</sup>	3.00
98	3.89	38.0	S <sub>3</sub>	96	3.89	37.3	S <sub>3</sub>	78	3.06	37.8	S <sub>1</sub> <sup>†</sup>	118 <sup>1</sup>	4.84	43.0	S <sub>1</sub> <sup>†</sup>	92	3.99
84	2.95	35.0	S <sub>1</sub> <sup>†</sup>	81	2.94	30.0	S <sub>1</sub> <sup>†</sup>	84	3.56	36.8	S <sub>1</sub> <sup>†</sup>	123 <sup>2</sup>	5.14	44.0	S <sub>1</sub> <sup>†</sup>	78	2.98
76	2.70	37.0	S <sub>1</sub> <sup>†</sup>	96	3.68	35.4	S <sub>1</sub> <sup>†</sup>	79	3.36	35.3	S <sub>2</sub>	115 <sup>2</sup>	4.29	43.7	S <sub>1</sub> <sup>†</sup>	84	3.41
78	2.67	36.0	S <sub>1</sub> <sup>†</sup>	80	2.51	34.8	S <sub>1</sub> <sup>†</sup>	90 <sup>1</sup>	3.14	36.0	S <sub>2</sub>	101 <sup>2</sup>	3.74	43.8	S <sub>1</sub> <sup>†</sup>	79	3.61
84	3.08	37.7	S <sub>1</sub> <sup>†</sup>	100 <sup>1</sup>	3.55	34.7	S <sub>2</sub>	94 <sup>1</sup>	3.68	32.8	S <sub>1</sub> <sup>†</sup>	89 <sup>1</sup>	3.23	43.4	S <sub>1</sub> <sup>†</sup>	106	3.92
76	3.08	35.5	S <sub>3</sub>	88	3.42	36.4	S <sub>3</sub>	89	3.10	34.4	S <sub>1</sub>	96 <sup>2</sup>	3.27	45.5	S <sub>1</sub> <sup>†</sup>	90	3.32
								95 <sup>1</sup>	3.61	33.4	S <sub>1</sub> <sup>†</sup>	140 <sup>2</sup>	4.69	46.5	S <sub>1</sub> <sup>†</sup>		
								80	2.40	36.5	S <sub>1</sub>	100 <sup>2</sup>	3.12	45.2	S <sub>1</sub> <sup>†</sup>		
94	3.53	36.3		99	3.66	35.6		86	3.28	35.6		109	4.14	44.0		89	3.50
±26	±1.09			±15	±0.70			±11	±0.52			±30	±1.20			±13	±0.40
																115	4.32
																	±0.90
																	43.5

$\frac{L}{B}$  = Lung wt. in grams;  $\frac{L}{V}$  = Lung wt. in grams  
 $\frac{B}{V}$  = Body wt. in grams;  $\frac{B}{V}$  = Vent. wt. in grams

respiration. D followed by number = Died at — hours of respiration.

<sup>1</sup> = Occasional hemorrhage, slight congestion.

<sup>2</sup> = Marked hemorrhage and congestion.

S followed by number = Killed at — hours of

ing box. The body and respiratory airway temperatures of both animals, in addition to the room and warming box temperatures, were recorded at 15-minute intervals. The state of the lungs at autopsy was assessed by lung to body weight and lung to ventricle weight ratios as well as by gross examination. Lung weights referred to herein represent combined weight of the lungs.

#### RESULTS AND DISCUSSION

The lung weight/body weight and lung weight/ventricle weight ratios shown in table 1 reveal that an occasional control animal, subjected to artificial respiration at 18 mm. Hg, develops significant pulmonary edema within 3 hours. Previously published observations from this laboratory (8) show that the normal lung weight/body weight ratio in 26 guinea pigs ranging in weight from 246 to 395 gm. with a mean weight of 310 is 0.0072 immediately after light anesthetization. Thus the three series of 'control' pigs in this study which were treated with positive pressure artificial respiration and had mean ratios of 0.0094, 0.0086 and 0.0089 showed evidence of moderate edema. The same inference can be drawn from the lung weight/ventricle weight data. In the previous study (8) this ratio was found to be 2.41 in the 26 animals killed immediately after anesthetization. The mean 'control' values in the present study all show some elevation above this figure. Thus the stresses of hyperthermia and humidified air are superimposed upon a condition which itself produces slight to moderate edema. The effects of the added stresses are evident in the differences between the control and experimental values in each series. It will be noted that this difference in Series I is  $5 \times 10^{-4}$  for the lung/body weight ratio, and 0.13 for the lung/ventricle weight. Neither of these differences has statistical significance, the respective *P* values according to Fisher's method being 0.45 and 0.75. On the other hand the differences between the control and experimental values in Series II are great, in the case of L/B the difference in the means shows  $P = 0.01$  and for L/V, *P* is between 0.02 and 0.05. Likewise in Series III for the difference in means for L/B,  $P < 0.01$  and for L/V,  $P < 0.02$ . Thus in both cases the experimental procedure increased lung weight significantly. There is not a significant difference between the two experimental groups in Series II and III. The morphologic observations upon the lungs showed that edema, congestion and hemorrhage contributed to the lung weight increases. The data at hand do not allow one to determine the precise fractions due to each, but the notations in table 1 indicate that in some cases there was massive edema with only slight congestion or hemorrhage.

It seems to be established that hyperthermia favors the production of the pulmonary lesions observed, and that the humidity of the inspired air is a factor of no great consequence. The mechanism by which hyperthermia may act to produce the above changes has not been elucidated. It may be pointed out that in the experiments on dogs, referred to above (2) measurements of pulmonary vascular pressures (9) showed that the pulmonary venous pressure rose most sharply when the body temperature was elevated. This finding would be consistent with the hypothesis that left ventricular failure was induced by hyperthermia. The pulmonary lesions could be accounted for on this basis.

## SUMMARY

Under the conditions studied, hyperthermia favored the production of pulmonary edema, hemorrhage and congestion in guinea pigs. The humidity of the inspired air was a factor of no great consequence in the production of pulmonary lesions in these studies.

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# PERSISTENT UNILATERAL RENAL HYPERTENSION IN THE RABBIT<sup>1</sup>

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SINCE the work of Goldblatt, the rôle of the kidney in hypertension has been extensively reinvestigated in various laboratory animals. The effect of hypertension per se on the kidney has received scant attention experimentally. If this were to be studied in animals with renal hypertension, it would be necessary to use animals with, what we have called, unilateral renal hypertension—that is hypertension due to the manipulation of one kidney or its blood supply without removing the other kidney. We are presenting the successful production of unilateral renal hypertension in the rabbit.

Most of the work in experimental renal hypertension has been done in the dog, and, unfortunately, in this species the manipulation of both kidneys is almost always necessary in order to get a persistent hypertension (1-5). Persistent unilateral renal hypertension has been produced in the rat (6-9). These experiments have been questioned by some because of the purported occurrence of 'spontaneous' renal disease (10, 11), and even 'spontaneous' hypertension (10) in this animal. Also, the reliability of the methods for determining blood pressure in these experiments is in some doubt (12-14). Unilateral renal hypertension has been reported in the rabbit (15-20), but this work has received scant attention. Possibly the inadequacy of the data pertaining to the persistence of the hypertension, the subjectivity of the blood pressure methods used, or the uncertainty of a truly unilateral origin of the renal hypertension accounts for the lack of recognition of these experiments (2, 3, 8, 9). At any rate, the apparent absence of spontaneous hypertension in the rabbit, suggested the latter as a suitable animal for this study.

## METHODS

*Production of Renal Hypertension.* We have produced persistent renal hypertension by the application of a pre-formed latex capsule to one kidney. Sobin (13) introduced the latex capsule for the purpose of producing hypertension in the rat by bilateral renal application; and Abrams and Sobin (21) did a few bilateral renal encapsulations in the rabbit but did not observe the blood pressure. They did note that the perinephritis due to latex in the rabbit was similar pathologically to the perinephritis due to latex, silk or cellophane in the rat. These foreign substances produce a fibrocollagenous perinephritic hull that apparently compresses the kidney through cicatricial contraction. We chose latex encapsulation instead of silk or cellophane encapsulation or instead of renal artery constriction, only because it appeared to be the technically simplest way to get hypertension in a large percentage of animals.

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Kidney-shaped glass forms were blown from tubing 1 cm. in external diameter. There was a very slight indentation at the 'hilum'. They were made in three sizes to simulate the kidneys of rabbits weighing approximately 4 to 6 pounds. The forms were dipped in liquid latex<sup>2</sup> two or three times at about one-hour intervals. Sometimes an additional layer of latex was painted on at the 'hilum'. The latex capsule was removed from the glass form by gradually working water under the capsule and then stretching it off. The capsules were then dried and powdered with talc to prevent adherence of opposing surfaces (the capsules were inverted during this procedure). When needed, they were washed with and sterilized in 70 per cent ethyl alcohol and were applied 'wet'.

Under ether anesthesia, the left kidney was exposed by an anterior (trans-peritoneal) or posterior (extra-peritoneal) approach; the adipose capsule and renal fascia were stripped off. The index and middle fingers of both hands supported the medial side of the kidney while the stretched capsule was slipped on with an inverting

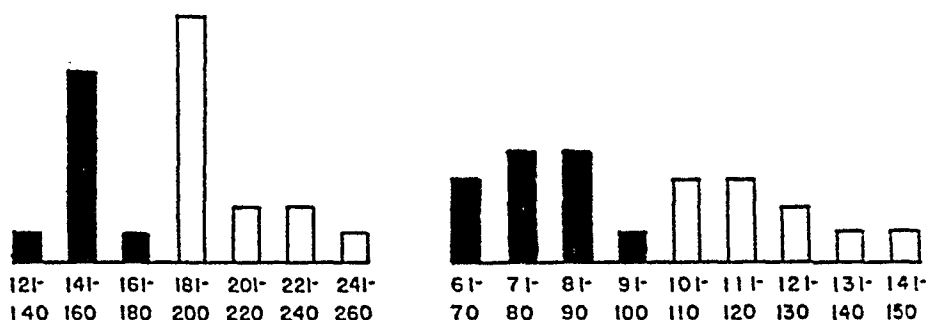


Fig. 1. (left) RELATIVE FREQUENCY (abscissa) of various levels of the highest systolic abdominal blood pressure in mm. Hg (ordinate) of 9 control animals (solid bars) and 15 rabbits that developed hypertension after the latex encapsulation of one kidney (open bars).

Fig. 2. (right) RELATIVE FREQUENCY (abscissa) of various levels of the highest mean ear blood pressure in mm. Hg (ordinate) of 12 control animals (solid bars) and 10 rabbits that developed hypertension after the latex encapsulation of one kidney (open bars).

maneuver by use of the thumbs. The capsules usually fitted a bit loosely, but a few fitted rather snugly. There was no constriction of the hilar structures by the stem or 'hilum' of the latex capsule at the time of application. The right kidney or its blood supply was not removed or manipulated.

*Blood Pressure Measurement.* Blood pressure was determined by two methods, an indirect and a direct. The former was the abdominal cuff method. Fahr (22) introduced the method, but McGregor (23) has modified and standardized it. The animal was tied down on its back and the cuff was wrapped around the abdomen; the systolic and diastolic blood pressures were determined by auscultation over the aorta under the distal edge of the cuff. In 1120 determinations in 84 normal rabbits, McGregor found the average systolic blood pressure to be 125 mm. Hg, with a range of 90 to 160 mm. Hg. Although this does not represent the true abdominal aorta blood pressure, it bears quite a constant relation to it (24). In 56 determinations in 14 control rabbits we noted a systolic blood pressure over 160 mm. only once, when it was 164 mm. Hg. Because we were desirous of studying animals that were truly hyper-

<sup>2</sup> The General Latex and Chemical Corporation, Cambridge, Mass.

tensive, rather than merely defining the lower limit of hypertensive blood pressures, we only considered a systolic abdominal blood pressure of 180 mm. Hg or more to be a 'hypertensive' reading.

In most of our animals, the blood pressure was also determined by a second method. The rabbit was placed in a simple warming chamber, and after the central artery of the ear became well dilated, arterial puncture was easily performed with a 22-gauge needle. The mean blood pressure was then observed on a small bore mercury manometer. The method was standardized on 12 normal rabbits; 29 determinations ranged from 60 to 93 mm. Hg. Landis, Montgomery and Sparkman (24)

TABLE 1

*Rabbit 187:* Latex capsule applied on July 9, 1948. Note spontaneous remission in hypertension after Aug. 30, '48.

Date:	7/24/48	8/14	8/19	8/28	8/30	9/23	10/7	10/18	11/9
Abdom. B. P.:	170/120	170/125		195/150		140/98			162/92
Ear B.P.:			105		122		84	79	

*Rabbit 153:* Latex capsule applied on Mar. 6, 1948. This animal was used for another experiment on May 5, '48.

Date:	3/8/48	3/13	3/20	3/26	4/1	4/3	4/13
Abdom. B.P.:	145/100	142/100	150/100	184/?	176/128	194/?	230/150
Date:		4/14	4/17	4/21	4/24	4/29	5/1
Abdom. B.P.:		208/154	198/146		216/154		204/168
Ear B.P.:				112		106	

*Rabbit 171:* Latex capsule applied May 6, '48. Animal was used for another experiment on July 21, '48. Note fluctuating nature of the hypertension.

Date:	5/11/48	5/22	5/25	6/4	6/7	6/23	6/25	7/13	7/16
Abdom. B.P.:		182/140		145/?		194/142		164/114	
Ear B.P.:	91		84		103		107		107

*Rabbit 174:* Latex capsule was applied June 8, '48. Animal is still under observation.

Date:	6/18/48	6/29	7/13	7/14	7/29	8/5	8/21	8/26
Abdom. B.P.:	148/114	164/128	204/160		245/196		215/165	205/155
Ear B.P.:				139		124		
Date:	8/30	9/23	10/7	10/18	11/2	11/18	1/26/49	
Abdom. B.P.:	240/160	194/?	204/164		198/158	186/150		
Ear B.P.:				132			109	

using a somewhat similar method, found a systolic blood pressure range of 70 to 98 mm. Hg. Again, we did not consider a mean ear blood pressure as 'hypertensive' unless it was at least 100 mm. Hg. Furthermore, when we speak of a 'hypertensive' animal, we are referring only to those that have had at least two hypertensive readings on different days by one or both of the above blood pressure methods.

#### RESULTS AND DISCUSSIONS

Twenty-two rabbits thus far have survived the latex encapsulation procedure. Fifteen of them or just over two thirds, have developed hypertension (figs. 1 and 2). The highest mean ear blood pressure we recorded was 148 mm. Hg, and the highest systolic abdominal blood pressure was 245 mm. Hg. The first hypertensive reading usually was noted between the 15th and 66th day after renal encapsulation, but in

one rabbit the first hypertensive reading did not occur until the 120th day after surgery, and in another rabbit it occurred as early as the 5th post-operative day (blood pressures were not taken during the first few post-operative days). In the latter rabbit, one would surmise that the latex capsule itself or its contained exudate, rather than a contracting perinephritic hull, compressed the kidney and so initiated the hypertension.

Nine of the 15 hypertensive rabbits were used for another experiment 18 to 111 days after their first hypertensive reading. Of the remaining 6, one died 47 days after the first hypertensive reading, 2 had a spontaneous and apparently permanent remission 11 to 34 days after the first hypertensive reading, and the other 3 are still exhibiting hypertension 115, 116 and 125 days after the first hypertensive reading. This represents a considerable period of time in the life of a rabbit, so we feel justified in referring to this as persistent unilateral renal hypertension. In table 1 we have included the protocols of 4 of the hypertensive rabbits, as representative of that group.

#### CONCLUSIONS AND SUMMARY

Persistent unilateral renal hypertension has been produced in the rabbit. A latex capsule was put on one kidney and the other kidney or its blood supply was not manipulated. This preparation may have usefulness in the study of the effects of hypertension per se on the normal kidney. One could also study the course of hypertension after the removal of the source in such a preparation. A simple method for obtaining repeated direct mean blood pressures in the unanesthetized rabbit has also been presented.

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# EFFECTS OF REMOVAL OF THE 'ISCHEMIC' KIDNEY IN RABBITS WITH UNILATERAL RENAL HYPERTENSION, AS COMPARED TO UNILATERAL NEPHRECTOMY IN NORMAL RABBITS<sup>1</sup>

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IN A previous paper (1) we presented a method for the production of *unilateral* renal hypertension, that is, hypertension due to unilateral renal or reno-vascular manipulation with the other kidney intact. It consisted of the latex encapsulation of one kidney. Blood pressure was determined by two methods, an indirect and a direct. The former was the abdominal cuff method, as modified by McGregor (2); he found the average systolic blood pressure to be 125 mm. Hg, with a range of 90 to 160 mm. Hg. In the second method, the rabbit was placed in a simple warming chamber, and after the central artery of the ear became well dilated, arterial puncture was easily performed with a 22-gauge needle. The mean blood pressure was then observed on a small bore mercury manometer. We found the normal range of blood pressure to be 60 to 93 mm. Hg. Hypertension, that is, two or more hypertensive readings, by either one or both of the blood pressure methods used, was produced in 15 of 22 rabbits. A 'hypertensive' reading was defined as a systolic blood pressure of at least 180 mm. Hg by the abdominal cuff method, or a mean blood pressure of at least 100 mm. Hg by our ear method in the central artery of the ear. It was noted that this hypertension may persist for a considerable period of time. Three rabbits still exhibited hypertension 115, 116, and 125 days after their first hypertensive reading; most of the others were used for the experiment to be presented shortly.

In the work presented in this paper we were interested in investigating whether the continued high blood pressure sets up mechanisms that tend to perpetuate the hypertension. In other words, is a vicious circle brought about? This can be readily studied in the rabbit with unilateral renal hypertension since the original cause can be removed at will and the animal studied thereafter.

## PROCEDURE

Our general plan of attack was to produce hypertension and then, after varying intervals, to remove the 'ischemic' kidney and follow the blood pressure thereafter. To serve as controls for this work, and because one investigator has reported that unilateral nephrectomy causes a rise in blood pressure in rabbits (3), we also subjected normal rabbits to unilateral nephrectomy and followed the blood pressure thereafter.

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*Effect of Unilateral Nephrectomy on the Blood Pressure of Normal Rabbits.* Nine normal rabbits were subjected to unilateral nephrectomy. Blood pressures were followed by the two blood pressure methods used in our previous paper; 33 of 34 determinations by the ear method, over a period up to 209 days after surgery, were normal. That is, they ranged from 62 to 90 mm. Hg, as compared to a range of 58 to 93 mm. Hg in 32 observations in our 12 normal controls (fig. 1). However, one ear blood pressure determination, in one of the unilaterally nephrectomized rabbits, was 109 mm. Hg, a hypertensive reading by our definition. Since the three previous and two subsequent ear blood pressure determinations in this rabbit were non-hypertensive, we do not classify this animal as one with hypertension. Further, none of these 9 unilaterally nephrectomized rabbits exhibited any hypertensive readings in 51 determinations by the abdominal cuff method; the range of systolic blood pressure was 120 to 164 mm. Hg.

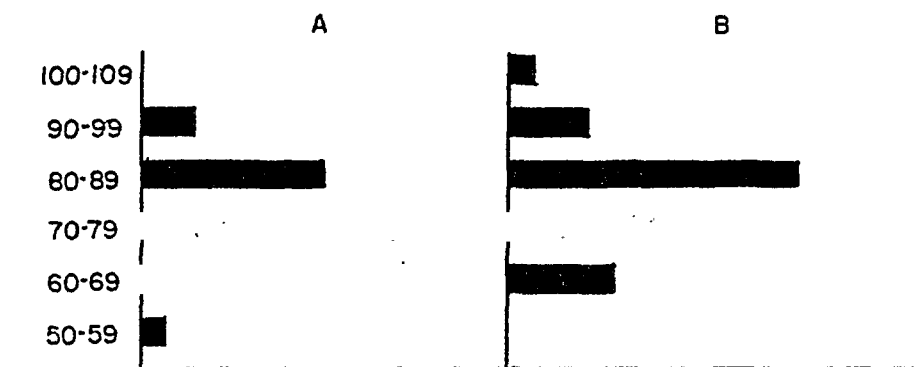


Fig. 1. RELATIVE FREQUENCY (abscissa) of various levels of mean ear blood pressure in mm. Hg (ordinate). A) 32 determinations in 12 normal rabbits; B) 34 determinations in 9 normal rabbits after unilateral nephrectomy.

*Effect of Removal of the 'Ischemic' Kidney on the Blood Pressure of Rabbits with Unilateral Renal Hypertension.* Seven rabbits with hypertension were subjected to removal of the encapsulated kidney 18 to 66 days after their first hypertensive reading. The hypertension was still present after that operation in 4 of the animals. It finally disappeared spontaneously 23, 48, and 152 days after the operation in 3 of these 4 rabbits, and one rabbit was used for another experiment 11 days after the operation. The blood pressure fluctuated considerably from hypertensive to non-hypertensive levels before it reverted to non-hypertensive or completely normal levels; this was especially true in the rabbit that had a persistence of hypertension for 152 days. The other 3 hypertensive rabbits, that were subjected to removal of the encapsulated kidney, did not exhibit any further hypertensive readings (the first reading was not taken until the third to fifth post-operative day). Even the latter three, however, exhibited blood pressures above the usual upper limits of normal for at least the first few post-operative weeks, i.e., abdominal systolic blood pressures of 160 to 180 mm. Hg.

There appeared to be a positive correlation between the height of the blood pressure and duration of hypertension after removal of the encapsulated kidney. Two of the 4 rabbits that exhibited this persistence of hypertension had one or two

mean ear blood pressures of at least 110 mm. Hg. Further, the latter 2 and another one of these 4 rabbits exhibited two to four systolic abdominal blood pressures of at least 200 mm. Hg. The blood pressures of the 3 rabbits that did not show a persistence of hypertension after the operation were all under the above values.

We have also checked these results in seven rabbits made hypertensive by partially constricting one renal artery with the other kidney intact (4, 5). The manipulated kidney was removed 3 to 80 days after the first hypertensive reading. The hypertension was still present after surgery in 3 of these 7 rabbits. These 3 animals remained hypertensive for at least 72, 103, and 103 days, when they were used for another experiment. In this group of 7 rabbits, one could not draw any conclusions about the relation of the level of the blood pressure to the persistence of hypertension after removal of the 'ischemic' kidney. Possibly, this is related to the

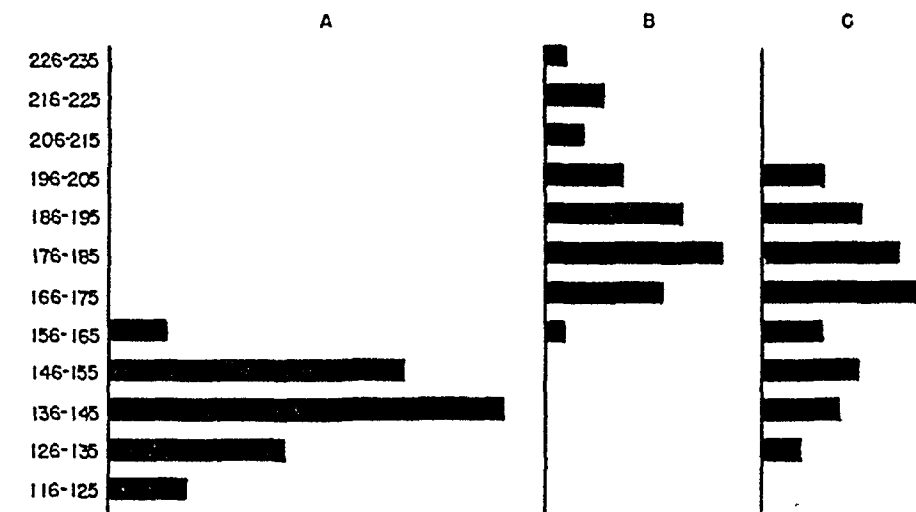


Fig. 2. RELATIVE FREQUENCY (abscissa) of various levels of systolic abdominal blood pressure in mm. Hg (ordinate). A) 51 determinations in 9 normal rabbits after unilateral nephrectomy; B) 33 determinations in 7 hypertensive rabbits; C) 37 determinations in the same 7 rabbits after removal of the 'ischemic' kidney (these animals remained hypertensive for various periods of time).

milder and more equal degree of hypertension that existed in this group. In considering the presence of any correlation between the duration of hypertension before removal of the 'ischemic' kidney and the persistence of hypertension after that operation, we can combine the findings in the two groups of rabbits. In 14 rabbits subjected to removal of the 'ischemic' kidney, in whom the duration of hypertension ranged from 3 to 80 days, there did not appear to be a notable positive correlation between the duration of hypertension before and the persistence of hypertension in 7 of the animals after that operation, except at the extremes in duration. In figure 2 and table 1 we have presented these data. The duration of hypertension is probably somewhat greater than as presented, because of the somewhat lengthy interval between blood pressure readings at times.

#### DISCUSSION

Similar results have been reported in the rabbit (3), and in the rat (6-9). In the dog, however, removal of the 'ischemic' kidney brings the blood pressure down

to normal within 24 hours (10-12). In man, the 'cure' of hypertension of supposed unilateral renal origin by the removal of the diseased kidney is still a controversial matter.

Friedman, Jarman, and Klemperer (7) were apparently the first investigators to show a persistence of hypertension after removal of the ischemic kidney in animals—the rat—with unilateral renal hypertension. Wilson and Byrom (6) have noted that removal of the ischemic kidney, in rats with unilateral renal hypertension, may revert the blood pressure to normal, even in those rats that have developed the malignant syndrome. Grollman (3), in 5 rabbits with unilateral renal hypertension, removed the manipulated kidney about 10 to 14 weeks after it was made ischemic by distortion with cotton tape; he noted a persistence of hypertension in all. He does not mention how long the hypertension had existed or how long these animals

TABLE I

RABBIT NO.	PREOPERATIVE DURATION OF HYPERTENSION	POST-OPERATIVE PERSISTENCE OF HYPERTENSION	RABBIT NO.	PREOPERATIVE DURATION OF HYPERTENSION	POST-OPERATIVE PERSISTENCE OF HYPERTENSION
	days	days		days	days
70	3	<22	175	33	<5
71	3	<17	153	35	48
60	4 <sup>1</sup>	<9	170	37	11 <sup>3</sup>
133	11	<4 <sup>4</sup>	147	52	<5
45	17 <sup>1</sup>	72 <sup>3</sup>	148	66	<3
176	18	23	89	76	103 <sup>3</sup>
150	32	152 <sup>2</sup>	92	80	103 <sup>3</sup>

<sup>1</sup> True duration of hypertension is probably somewhat greater than this, because of the long interval between blood pressure readings in these animals.

<sup>2</sup> The blood pressure was very fluctuant with frequent normal readings.

<sup>3</sup> Rabbit 170 was used for another experiment at that time. Rabbits 89 and 92 were probably still hypertensive when used for another experiment 10 days later. Rabbit 45 was not followed thereafter.

<sup>4</sup> Not followed adequately.

were followed after removal of the 'ischemic' kidney. By the same method of determining blood pressure, he also observed hypertension after unilateral nephrectomy in normal rabbits, a result in disagreement with the work of most investigators, and with our results. Pickering (13) found no elevation of blood pressure after removal of one or both kidneys in the rabbit. Braun-Menendez *et al.* (11) state that it has been shown that unilateral nephrectomy is not followed by any appreciable change in blood pressure in any tested animal. Pickering (13) has noted that 'bilateral' renal hypertension of at least 7 weeks' duration, in the rabbit, persists even after both manipulated kidneys are out (in the several days the animals survive). In rabbits, with 'bilateral' renal hypertension of up to 8 days' duration, there was no persistence of hypertension; it disappeared in a few hours after the second kidney was removed, just as he noted to be true after cessation of a prolonged renin injection.

Our results support the view (3, 6-9, 13, 15) that there is a 'change of mechanism' in the course of renal hypertension. Possibly a continued hypertension can

produce effects which will maintain the hypertension after the original cause is removed. A high blood pressure that has lasted for some time does produce changes in the body, although undoubtedly most of these effects are unknown to us. However, in the category of anatomical changes, we might cite the hypertrophy of the left ventricle. In the category of physiological changes we may cite the normal pulse rate in the presence of a raised blood pressure. If we raise the blood pressure of a normal rabbit (by compression of the abdominal aorta) to a hypertensive level, the heart rate becomes slowed. The heart rate of the hypertensive rabbit will respond to a rise of blood pressure above its control level, i.e. a slowing following compression of the abdominal aorta. It would seem that this reflex mechanism might be dulled to the ordinary blood pressure stimulus and needed a more pronounced stimulus, or we may look on it as a change in the 'set' of a regulatory mechanism.

These known effects of a continued high blood pressure probably play a rôle in the persistence of hypertension after removal of the ischemic kidney. Undoubtedly other factors are involved. This self-perpetuation of some disturbance in function after removal of the inciting cause is not unknown in physiology. There is much evidence to support the concept that long continued induced hyperglycemia will result in a hyperglycemia that persists after the animal is put back on a normal regime. It is believed that the high sugar level over-strains the islet tissue of the pancreas so that thereafter it functions inadequately. Adiposity tends to perpetuate itself by limiting the activity of the individual. A weakened heart action is liable to produce a vicious circle since it leads to reduced coronary blood flow. In these examples the mechanisms bringing about the self-perpetuation are quite different and may only be indirectly related to the characteristic itself, as in the case of adiposity.

The non-operated kidney may play an important rôle in this self-perpetuation. It may be affected by the high blood pressure per se so that its functioning is so altered that it aggravates the pressure raising process by secreting a pressor substance or by some other means. On the other hand, the changes produced on the experimental kidney may in some specific manner result in an alteration of the other kidney by some mechanism analogous to sympathetic ophthalmia. Only in this latter case would our experimental method lose some of its value as a research tool in the study of hypertension.

#### CONCLUSIONS AND SUMMARY

None of the 8 normal rabbits subjected to unilateral nephrectomy developed hypertension. Seven of the 14 rabbits with unilateral renal hypertension showed a persistence of hypertension for some time after removal of the 'ischemic' kidney.

We believe that a long continued high blood pressure is a characteristic which tends to be self-perpetuating. This might be due to overstraining or 'dulling' of a regulatory mechanism, or might be due to indirect effects, such as renal damage or widespread vascular damage.

Since our work, and more especially work in the rat, shows that removing the ischemic kidney in unilateral renal hypertension may not 'cure' the hypertension perhaps it is not safe to assume that man will be cured of supposed unilateral renal

hypertension by the removal of the involved kidney. This operation was founded in part on the observation that in the dog, removal of the ischemic kidney, during the course of the temporary unilateral renal hypertension that can be produced in that animal, promptly brought the blood pressure down to normal.

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# INFLUENCE OF SODIUM LOAD ON SODIUM EXCRETION<sup>1</sup>

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THE relation of sodium to heart failure and arterial hypertension has emphasized the need for more complete knowledge of the factors which regulate its excretion. Existing data relative to overall sodium metabolism have been summarized in recent reviews and monographs (1). The purpose of the present study was to measure the alterations in renal function which follow the administration of sodium chloride and to determine the mechanism through which increased sodium intake alters sodium output.

## METHODS AND MATERIALS

Renal sodium excretion is presumably determined by the relation between the amount of sodium filtered by the glomeruli and the fraction of filtered sodium which escapes tubular reabsorption. On the assumption that glomerular urine is an ultrafiltrate of plasma, the magnitude of sodium filtration was estimated in these studies by the expression:

$$\text{GFR}_{\text{Na}} = \text{GFR} \times \text{B}_{\text{Na}}$$

Where:  $\text{GFR}_{\text{Na}}$  = Sodium filtration rate (mEq/min.);

$\text{GFR}$  = Glomerular filtration rate (cc/min.) as measured by the mannitol technique (2);

$\text{B}_{\text{Na}}$  = Plasma sodium concentration (mEq/cc.) as measured by the uranium acetate method (3).

The influence of tubular activity on sodium excretion was estimated by the expression:

$$\text{TRF}_{\text{Na}} = \frac{\text{U}_{\text{Na}} \cdot \text{V}}{\text{GFR}_{\text{Na}}} \times 100$$

Where:  $\text{TRF}_{\text{Na}}$  = Sodium tubular rejection fraction (mEq. sodium excreted per 100 mEq. filtered).

$\text{U}_{\text{Na}}$  = Urinary sodium concentration (mEq/cc.).

$\text{V}$  = Urine flow (cc/min.).

$\text{GFR}_{\text{Na}}$  = Sodium filtration rate (mEq/min.).

Renal plasma flow was measured by the para-aminohippurate technique (4).

The relative importance of the glomerular and tubular mechanisms in compensating for changes in sodium intake was studied in a series of 12 normal dogs. The weights of the animals varied between 8 and 26 kg. Surface area was calculated by the formula:  $\text{Wt}_{\text{kg}}^{2/3} \times 0.107 = \text{m}^2$ .

On the preceding day 50 cc. of water per kilogram were given by stomach tube. This dose was repeated about 15 hours before the experiment. Anesthesia was accomplished with dial urethane<sup>2</sup>, 0.7 cc/kg. given intraperitoneally. Blood pressure was recorded continuously from the left common carotid artery through a cannula attached to a mercury manometer. Electrocardiograms were recorded from subcutaneous needles inserted in the right upper and left lower extremities. All blood

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samples were obtained from the femoral artery through an inlying needle. Infusions were made into the left jugular vein by a constant speed infusion pump. Urine was collected through an indwelling soft rubber catheter. The bladder was washed out with distilled water after each collection and emptied completely by blowing out with air.

Basal renal functional values were determined for each animal during two or more 30-minute control clearance periods. Sodium loads were then imposed by the intravenous administration of test solutions of sodium chloride which ranged in concentration from 0.86 to 30 per cent. The test solutions were infused at constant speeds for periods of 1 to 10 hours. Rates of administration and the values which were measured and derived are indicated in table 1. All chemical analyses were done in duplicate. Pre-injection samples were used for blank determinations.

## RESULTS

*Survival.* Animals infused at rates in excess of 13 milliequivalents of sodium per minute per square meter died at the end of 30 to 90 minutes. Death was preceded by sudden decrease in heart rate, disappearance of the P wave, increased amplitude of the T wave, and development of ventricular premature contractions. This phase was followed by ventricular asystole or fibrillation with rapidly fatal termination. Animals injected at rates less than 8.5 mEq. Na/min/m<sup>2</sup> outlived periods of observation from 3½ to 10 hours in duration.

*Sodium Excretion and Tubular Rejection Fraction.* The time until maximum output of sodium varied from 30 minutes to 8 hours after the start of infusion, and was related in general to the rate of sodium administration (table 1 and fig. 1). The maximum excretory rates were 40 to 500 times higher than the basal levels, depending upon the conditions of the experiment as discussed below.

In each animal the changes in sodium excretion were paralleled by corresponding alterations in the sodium tubular rejection fraction (fig. 2). This relationship appeared to be independent both of the concentration of sodium chloride solution infused and of the plasma sodium level. The maximum tubular rejection fraction observed represented approximately 42 per cent of the sodium filtered.

*Glomerular Filtration Rate.* Infusion of sodium chloride solutions did not produce consistent changes in glomerular filtration rate. In 5 animals infusion of the test solution was followed by a fall in filtration although sodium excretion increased. In those cases in which the filtration rate rose its highest level never exceeded twice the control value. In no instance did the greatest output of sodium occur during the period of maximum glomerular filtration.

*Renal Plasma Flow and Filtration Fraction.* Administration of salt solutions consistently resulted in a rise in renal plasma flow, which in some instances attained a value two and one-half times the control figure. Due to the magnitude of this change, the filtration fraction fell below the basal value in 5 experiments.

The rise in plasma flow usually occurred early and preceded the highest level of sodium output. As a consequence, a sustained or increasing rate of sodium excretion was often manifested in the face of a falling renal plasma flow (table 1, animals 1, 2, 5, 6, 7).

*Water Excretion.* The volume of urine flow increased concurrently with the rise in sodium excretion. The increase was due almost entirely to diminished tubular reabsorption of water, rather than to a change in glomerular filtration rate.

TABLE I

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
PERIOD	INJECTION RATE		URINE	GFR	H <sub>2</sub> O BALANCE	RPF	FF	UNa	UNa·V	GFRNa	TRFNa	Na BALANCE	EXTRA-CELLULAR Na	Na SPACE	Na CHANGE	CELL. H <sub>2</sub> O CHANGE	HEMATOCRIT	MEAN BP		
	Na	H <sub>2</sub> O																		
		mEq/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	%	mEq/cc.	mEq/min/m <sup>2</sup>	mEq/cc.	mEq/min/m <sup>2</sup>	mEq/m <sup>2</sup>	mEq/m <sup>2</sup>	cc/m <sup>2</sup>	cc/m <sup>2</sup>	cc/m <sup>2</sup>	Vol. %	mm. Hg	
Dog 1, female; 0.445 sq. m. surface area																				
Control Average (2 x 30')			0.66	49	1.35	131	37	0.038	0.020	0.155	7.65	0.27	540	3453						
T <sub>1</sub> (30')	3.90	2.29	0.58	42	1.40	155	27	0.160	0.094	0.137	5.74	1.64	653	4312	859	-832				138
T <sub>2</sub> (60')	3.90	2.29	3.28	37	8.78	130	29	0.171	0.562	0.174	6.48	8.97	846	4630	1177	-1236				115
T <sub>3</sub> (60')	3.90	2.29	4.16	48	8.77	203	148	0.173	0.720	0.191	9.13	7.90	1035	51308	1678	-1881				116
T <sub>4</sub> (60')	3.90	2.29	2.59	34	7.62	247	94	0.287	0.743	0.211	7.13	10.40	1219	5520	2067	-2314				106
Dog 2, male; 0.459 sq. m. surface area																				
Control Average (2 x 30')			1.15	120	0.96	355	34	0.086	0.098	0.152	18.3	0.54								
T <sub>1</sub> (30')	3.89	2.27	1.71	103	1.65	405	25	0.220	0.376	0.141	14.5	2.58								130
T <sub>2</sub> (60')	3.89	2.27	4.49	106	4.24	171	364	0.299	1.34	0.178	18.8	7.12								122
T <sub>3</sub> (60')	3.89	2.27	4.29	119	3.60	315	280	0.207	1.27	0.186	22.0	5.77								117
T <sub>4</sub> (60')	3.89	2.27	4.51	76	5.90	487	242	0.317	1.43	0.207	15.8	9.02								113
Dog 3, male; 0.658 sq. m. surface area																				
Control Average (2 x 30')			0.52	72	0.72	208	34	0.067	0.036	0.152	11.0	0.32	822	5400						
T <sub>1</sub> (30')	35.7	6.94	10.03	82	12.30	112	255	0.230	2.30	0.189	15.5	14.85	1841	8271	2871	-2083				143
T <sub>2</sub> (30')	35.7	6.94	26.90	71	31.90	427	259	0.300	6.79	0.227	16.3	41.80	2543	9587	4187	-4614				154
Dog 4, male; 0.652 sq. m. surface area																				
Control Average (2 x 30')			0.76	62	0.88	190	33	0.060	0.047	0.170	10.5	0.45	1024	6010						
T <sub>1</sub> (26')	72.2	14.05	8.59	55	15.54	81	226	0.216	2.11	0.261	14.4	14.60	2620	6771	761	-680				108
																				60

Dog 5, female; 0.534 sq. m. surface area

Control Average (2 x 30')	17.1	3.74	0.54	91	0.61	227	37	0.013	0.0032	0.154	14.0	0.04	358	1050	6820	87	145
T <sub>1</sub> (20')	17.1	3.74	5.90	82	7.16	-75	33	0.103	1.14	0.180	15.6	7.32	484	1378	6658	-162	183
T <sub>2</sub> (12')	17.1	3.74	13.27	75	17.61	-216	41	0.204	2.71	0.219	16.9	16.45		1534	6836	-232	115

Dog 6, female; 0.417 sq. m. surface area

Control Average (2 x 30')	14.5	8.48	0.52	84	0.61	230	39	0.016	0.009	0.141	11.8	0.08		607	4310		123
T <sub>1</sub> (20')	14.5	8.48	12.60	77	16.40	65	29	0.184	0.603	0.148	11.4	5.31	259	866	5310	1000	101
T <sub>2</sub> (20')	14.5	8.48	15.00	68	21.93	-35	25	0.194	2.46	0.196	15.0	16.30	512	1110	5363	1053	94
T <sub>3</sub> (20')	14.5	8.48	15.83	58	27.50	-201	23	0.234	3.51	0.226	15.4	22.75	714	1321	5027	1317	96
T <sub>4</sub> (20')	14.5	8.48	7.20	27	26.55	-371	21	0.254	4.01	0.250	14.4	27.95	926	1533	5688	1378	103
T <sub>5</sub> (7')	14.5	8.48				-390		0.399	2.22	0.278	7.52	29.50	1005	1612	5661	1351	71

Dog 7, male; 0.681 sq. m. surface area

Control Average (2 x 30')	8.30	4.84	0.49	74	0.67	301	25	0.043	0.213	0.157	11.7	0.14		778	4957		154
T <sub>1</sub> (20')	8.30	4.84	5.33	83	6.46	54	26	0.227	0.281	0.159	12.5	1.34	159	937	5555	598	160
T <sub>2</sub> (20')	8.30	4.84	11.73	87	13.49	11	25	0.232	1.24	0.177	14.6	8.48	269	1047	5735	778	146
T <sub>3</sub> (20')	8.30	4.84	21.44	95	22.50	-479	20	0.244	2.87	0.187	16.3	17.62	387	1165	6037	1080	140
T <sub>4</sub> (20')	8.30	4.84	22.50	78	28.81	-828	18	0.246	0.926	0.198	18.9	29.50	477	1255	6014	1057	129
T <sub>5</sub> (20')	8.30	4.84	16.15	70	23.16	-1057	21	0.261	1.53	0.216	16.9	34.90	542	1320	5863	906	118
T <sub>6</sub> (20')	8.30	4.84	14.83	70	21.13	-1270	21	0.292	4.72	0.233	16.2	29.05	632	1410	6049	1092	102
T <sub>7</sub> (20')	8.30	4.84	11.74	71	16.60	-1488	23	0.290	4.30	0.234	16.4	26.15	717	1495	6231	1274	94
T <sub>8</sub> (20')	8.30	4.84	11.31	59	19.11	-1622	28	0.335	3.94	0.247	17.5	22.55	698	1476	5772	815	96
T <sub>9</sub> (20')	8.30	4.84	6.02	25	23.70	-1652	29	0.314	3.55	0.264	15.7	22.65	810	1588	5954	897	122
T <sub>10</sub> (20')	8.30	4.84					25	0.332	2.00	0.277	7.04	28.42	933	1711	6053	1096	158

Dog 8, female; 0.410 sq. m. surface area

Control Average (2 x 30')	0.596	4.06	0.97	69	1.41	149	43	0.003	0.003	0.156	10.8	0.02		690	4423		147
T <sub>1</sub> (60')	0.596	4.06	0.68	71	0.97	173	41	0.009	0.006	0.155	11.0	0.06	31	721	4507	84	148
T <sub>2</sub> (60')	0.596	4.06	0.51	62	0.83	349	44	0.008	0.004	0.164	10.1	0.04	60	751	4628	205	143
T <sub>3</sub> (60')	0.596	4.06	0.73	69	1.07	557	52	0.011	0.008	0.160	10.9	0.07	91	781	5004	581	136
T <sub>4</sub> (60')	0.596	4.06	1.34	78	1.73	619	54	0.030	0.041	0.152	11.8	0.33	118	809	5407	984	136
T <sub>5</sub> (60')	0.596	4.06	1.83	79	2.31	756	41	0.057	0.105	0.146	11.4	0.92	145	835	5716	1293	128
T <sub>6</sub> (60')	0.596	4.06	2.37	56	4.22	827	26	0.072	0.220	0.146	8.26	2.07	166	856	5625	1202	130
T <sub>7</sub> (60')	0.596	4.06	1.51	98	1.54	944	54	0.105	0.107	0.160	15.7	1.01	186	876	5630	1207	140
T <sub>8</sub> (60')	0.596	4.06	1.51	117	1.29	1075	58	0.067	0.101	0.151	17.7	0.57	213	903	5986	1563	152
T <sub>9</sub> (60')	0.596	4.06	1.29	140	0.92	1201	68	0.047	0.060	0.131	21.1	0.28	238	928	6187	1764	156
T <sub>10</sub> (60')	0.596	4.06	1.10	123	0.89	1328	63	0.080	0.088	0.149	18.3	0.48	260	950	6390	1967	148

TABLE 1—Continued

PERIOD	INJECTION RATE		URINE	GFR	H <sub>2</sub> O BAL-ANCE	RPF	FF	UNa	UNa·V	BNa	GFRNa	TRFNa	Na BAL-ANCE	EXTRA-CELL-LAR Na	Na SPACE	Na SPACE CHANGE	CELL. H <sub>2</sub> O CHANGE	HEMATOCRIT	MEAN BP
	Na	H <sub>2</sub> O																	
	mEq/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc/min/m <sup>2</sup>	cc./excr/100 cc. filtered	cc/min/m <sup>2</sup>	%	mEq/cc.	mEq/min/m <sup>2</sup>	mEq/cc.	mEq/min/m <sup>2</sup>	mEq/100 mEq filtered	mEq/m <sup>2</sup>	mEq/m <sup>2</sup>	cc/m <sup>3</sup>	cc/m <sup>2</sup>	cc/m <sup>2</sup>	Vol. %	mm. Hg
<i>Dog 9, male; 0.434 sq. m. surface area</i>																			
Control Average (2 x 30')																			
T <sub>1</sub> (60')	2.02	10.83	0.91	71	1.26	215	38	0.013	0.012	0.146	10.4	0.12	178	661	457	1103	45	60	101
T <sub>2</sub> (60')	2.02	10.83	1.15	70	1.67	254	27	0.0778	0.090	0.146	10.2	0.88	343	840	5630	2130	17	55	108
T <sub>3</sub> (60')	2.02	10.83	2.77	69	4.04	2113	31	0.144	0.401	0.132	10.4	3.84	524	1001	6657	3332	-267	116	120
T <sub>4</sub> (60')	2.02	10.83	4.69	68	6.83	3065	30	0.136	0.635	0.149	10.3	6.21	697	1186	7859	4301	-386	38	131
T <sub>5</sub> (60')	2.02	10.83	5.53	72	7.75	3915	31	0.116	0.643	0.152	10.9	5.01	875	1359	8828	5960	-1045	37	143
T <sub>6</sub> (60')	2.02	10.83	3.76	67	5.51	4915	34	0.124	0.465	0.155	10.4	4.46	1054	1537	10487	7217	-1352	44	120
T <sub>7</sub> (60')	2.02	10.83	4.45	83	5.35	5805	41	0.133	0.591	0.139	11.6	5.13	1200	1716	11744	8337	-1732	37	123
T <sub>8</sub> (60')	2.02	10.83	4.24	74	5.74	6595	45	0.122	0.517	0.132	11.3	4.60	1361	12854	12854	9989	-2614	36	132
T <sub>9</sub> (60')	2.02	10.83	4.38	69	6.36	7375	37	0.121	0.533	0.139	9.00	5.92	1352	2013	14516	9989			
<i>Dog 10, female; 0.474 sq. m. surface area</i>																			
Control Average (2 x 30')																			
T <sub>1</sub> (60')	2.67	18.1	0.41	89	0.46	171	52	0.043	0.017	0.138	12.2	0.14	139	807	5833	1092	-191	49	125
T <sub>2</sub> (60')	2.67	18.1	3.18	90	3.23	233	42	0.120	0.383	0.137	13.5	2.81	350	945	6925	1092	-391	41	114
T <sub>3</sub> (60')	2.67	18.1	8.80	99	8.90	1511	26	0.117	1.03	0.137	13.5	7.60	279	1056	7738	1905	-453	36	118
T <sub>4</sub> (60')	2.67	18.1	12.69	96	13.20	1667	24	0.139	1.76	0.137	13.1	13.79	210	1086	7953	2120	-556	30	116
T <sub>5</sub> (60')	2.67	18.1	12.25	93	13.21	2044	30	0.135	1.66	0.137	12.7	13.10	345	1151	8433	2600	-502	30	136
T <sub>6</sub> (60')	2.67	18.1	11.45	96	11.79	2534	27	0.127	1.45	0.137	13.1	11.10	417	1223	8959	3126	-502	31	124
T <sub>7</sub> (60')	2.67	18.1	10.75	139	7.74	2884	49	0.133	1.44	0.137	19.0	7.56	491	1298	9597	3674	-790	35	133
T <sub>8</sub> (60')	2.67	18.1	8.85	102	8.55	3364	33	0.140	1.22	0.137	14.0	8.73	568	1374	10064	4231	-867	35	132
T <sub>9</sub> (60')	2.67	18.1	7.23	121	5.08	3994	46	0.113	0.821	0.137	16.5	4.97	672	1478	10838	4995	-1001	30	146

Dog 11, male; 1.06 sq. m. surface area

Control Average (2 x 30')	1.26	8.50	0.55	59	1.06	144	40	0.022	0.012	0.158	9.65	0.14	68	1409	8000	428	-272	65	156
T <sub>1</sub> (60')	1.26	8.50	1.11	59	1.00	403	37	0.021	0.033	0.158	9.30	0.25	138	1476	9328	428	-272	60	158
T <sub>2</sub> (60')	1.26	8.50	2.74	97	2.82	711	58	0.032	0.086	0.158	15.3	0.56	138	1547	9383	933	-265	63	158
T <sub>3</sub> (60')	1.26	8.50	3.63	82	4.40	1008	48	0.057	0.209	0.155	12.7	1.60	202	1610	10173	1273	-265	55	158
T <sub>4</sub> (60')	1.26	8.50	3.52	68	5.18	1273	37	0.090	0.348	0.162	11.0	3.20	250	1659	10199	1299	-26	56	158
T <sub>5</sub> (60')	1.26	8.50	3.76	84	4.35	1542	44	0.126	0.472	0.166	14.0	3.40	295	1703	10175	1275	267	55	159
T <sub>6</sub> (60')	1.26	8.50	2.24	57	3.02	1030	45	0.157	0.352	0.169	9.70	3.70	350	1759	10335	1635	295	54	156
T <sub>7</sub> (60')	1.26	8.50	2.69	67	4.07	2267	41	0.157	0.422	0.166	11.0	3.80	398	1807	11171	2271	-4	51	161
T <sub>8</sub> (60')	1.26	8.50	4.67	93	5.01	2491	42	0.150	0.700	0.158	14.8	4.70	431	1839	11049	3049	-558	47	158

Dog 12, female; 0.531 sq. m. surface area

Control Average (3 x 30')	5.30	7.56	0.87	66	1.28	233	28	0.0069	0.006	0.145	9.87	0.06	136	616	4240	512	-414	59	130
T <sub>1</sub> (20')	5.30	7.56	0.75	75	1.00	98	34	0.120	0.090	0.150	11.6	0.83	136	742	4752	512	-414	54	130
T <sub>2</sub> (20')	5.30	7.56	4.87	97	5.02	106	40	0.205	1.00	0.164	16.5	6.30	226	842	5028	788	-682	54	135
T <sub>3</sub> (20')	5.30	7.56	13.11	120	10.91	-57	39	0.183	2.40	0.170	21.1	12.00	294	910	5079	839	-896	51	131
T <sub>4</sub> (20')	5.30	7.56	17.44	139	12.58	-291	47	0.201	3.50	0.186	27.0	13.60	348	964	5179	939	-1233	54	127
T <sub>5</sub> (20')	5.30	7.56	17.44	136	11.19	-541	64	0.183	3.20	0.186	30.3	11.00	402	1018	5469	1229	-1770	52	114
T <sub>6</sub> (20')	5.30	7.56	17.44	140	12.46	-757	53	0.207	3.60	0.186	27.3	13.80	465	1081	5781	1541	-2298	53	102
T <sub>7</sub> (20')	5.30	8.10	8.10	68	11.91	-806	55	0.210	1.70	0.188	13.3	13.00	555	1171	6176	1936	-2742	55	85
T <sub>8</sub> (20')	5.30	7.56	10.13	105	9.62	-893	42	0.207	2.10	0.193	21.1	8.70	644	1260	6168	2158	-3051	51	76
T <sub>9</sub> (20')	5.30	7.56	11.86	109	10.91	-972	43	0.207	2.10	0.204	23.0	8.70	644	1260	6168	2158	-3051	50	65
T <sub>10</sub> (20')	5.30	7.56	9.39	90	11.05	-1055	48	0.207	2.10	0.210	19.6	8.70	644	1260	6168	2158	-3051	47	61

A comparison of the sodium and water rejection fractions showed them to be correlated (fig. 3). During control periods the rejection fraction for water exceeded that for sodium several fold. When infusion of the test solution was begun both fractions increased. The sodium rejection fraction, however, rose relatively more rapidly to reach a limiting value approximately equal to that for water. When this limiting value had been attained, further increases in the sodium and water rejection fractions were linearly related regardless of the concentration of the test solution infused or the magnitude of sodium excretion.

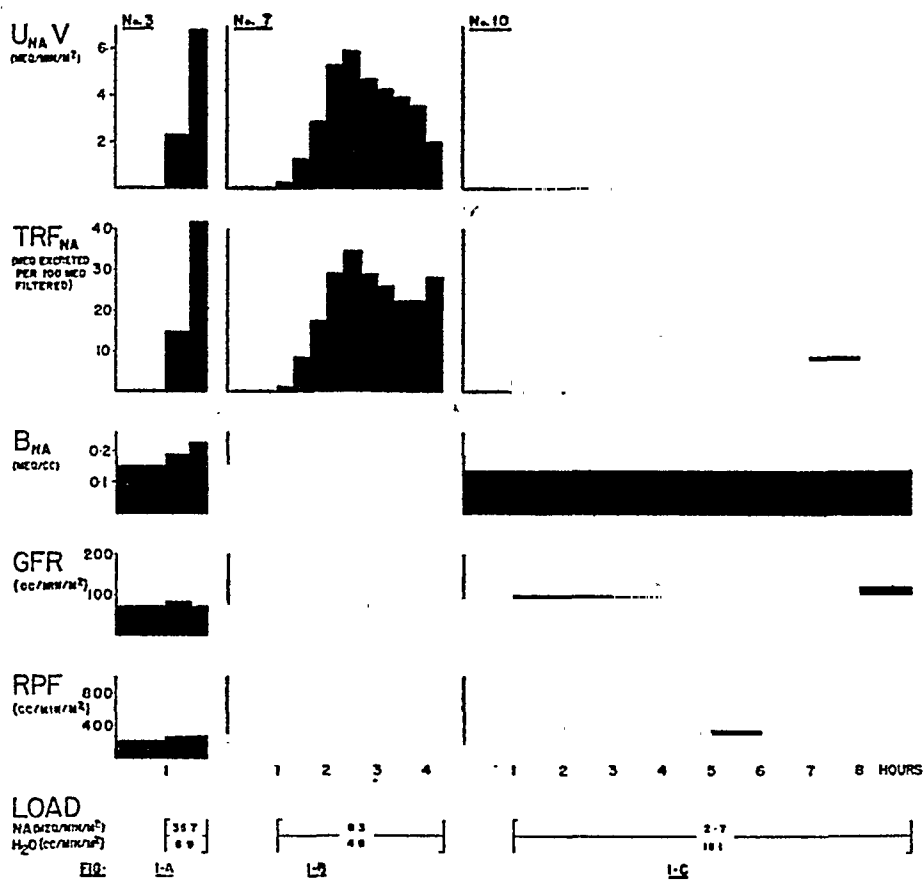


Fig. 1a, b, c. RELATION OF SODIUM EXCRETION to sodium tubular rejection fraction, plasma sodium concentration, glomerular filtration rate and renal plasma flow under varying conditions of sodium load.

By definition, the relation between the sodium and water rejection fractions should be reflected in the ratio of the corresponding urine and plasma sodium concentrations.<sup>3</sup> A comparison of the two latter parameters over the entire group of experiments (fig. 4) showed that the sodium concentration in the urine rose with infusion of the test solution to reach a limiting value which approximated the plasma concentration. Thereafter a further increase in urinary sodium concentration was obtained only in the presence of an equivalent rise in the plasma sodium level.

$$^3 \text{TRF}_{\text{Na}} = \frac{U_{\text{Na}} \cdot V}{\text{GFR} \times B_{\text{Na}}} \times 100; \quad \text{and: } \text{TRF}_{\text{H}_2\text{O}} = \frac{V}{\text{GFR}} \times 100; \quad \text{whence: } \frac{\text{TRF}_{\text{Na}}}{\text{TRF}_{\text{H}_2\text{O}}} = \frac{U_{\text{Na}}}{B_{\text{Na}}}$$

## DETERMINANTS OF SODIUM EXCRETION RATE

A number of associated factors were studied in an effort to determine the intermediate steps by which sodium administration provoked a rise in sodium excretion:

*Plasma Sodium Concentration.* No consistent relationship was established between sodium excretory rate and plasma sodium concentration. Infusion of isotonic solution produced marked increases in sodium excretion with little alteration in plasma sodium level (fig. 1 C). Comparisons between animals to which hypertonic solutions were administered failed to demonstrate a correlation between the rate of sodium excretion and the plasma sodium level. Serial observations in the same

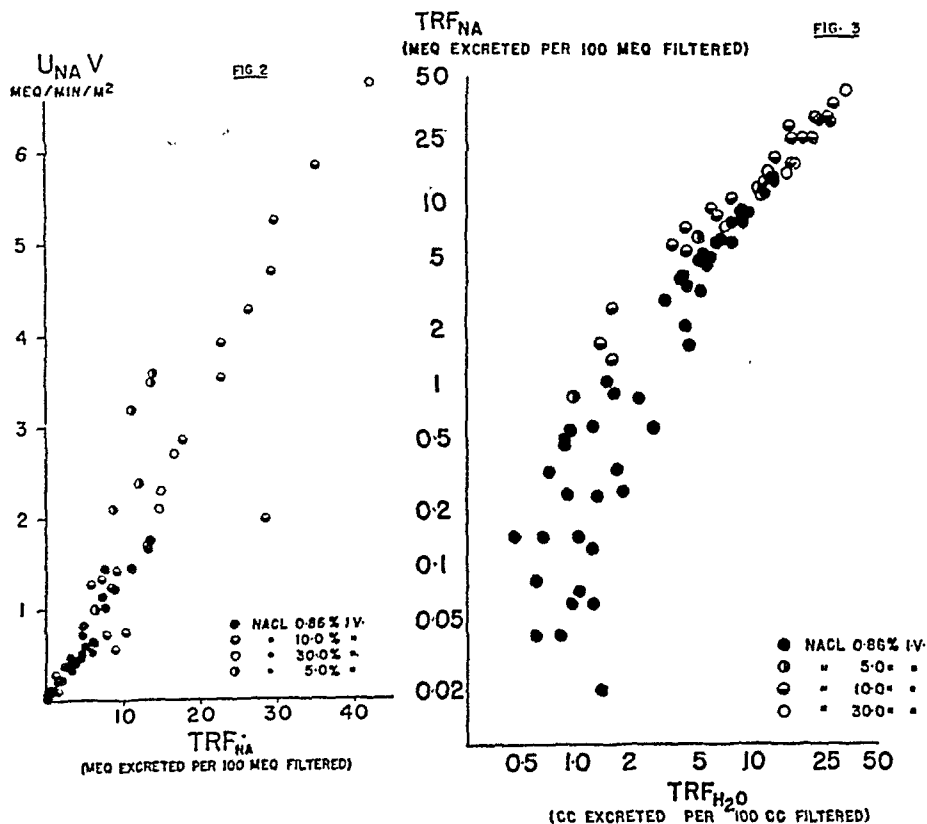


Fig. 2. RELATION OF SODIUM EXCRETION TO sodium tubular rejection fraction.

Fig. 3. RELATION OF SODIUM AND WATER tubular rejection fractions under sodium load.

animal showed that the maximum sodium excretory rate often developed prior to the attainment of the maximum plasma concentration. In such instances, the sodium output entered a declining phase while the plasma sodium level was still rising (fig. 1 B, table 1, animals 6, 7, 12).

*Sodium Balance, Extracellular Sodium Mass and the Sodium Space.* Three successive measurements of the basal extracellular fluid volume were made during the control periods by the mannitol technique (5). The average of these values was used to calculate the basal quantity of extracellular sodium by means of the expression:

$$XC_{Na} = XCV \times B_{Na}$$

Where:  $XC_{Na}$  = Extracellular sodium mass (mEq/m<sup>2</sup>);

$XCV$  = Extracellular fluid volume (cc/m<sup>2</sup>);

$B_{Na}$  = Plasma sodium concentration (mEq/cc.).

The sum of the basal extracellular sodium mass and the net sodium exchange at the end of any clearance period subsequent to the control period was taken to represent the total extracellular sodium at that time. This latter figure was divided by the corresponding plasma sodium concentration to estimate the volume of the sodium space, assuming uniform extracellular distribution of the infused sodium.

On the basis of these computations, it appeared that the continuous administration of sodium chloride solution, regardless of rate or concentration, resulted in a progressive increase in the values for extracellular sodium mass and sodium space during periods of observation up to 10 hours (table 1, columns 16, 17).

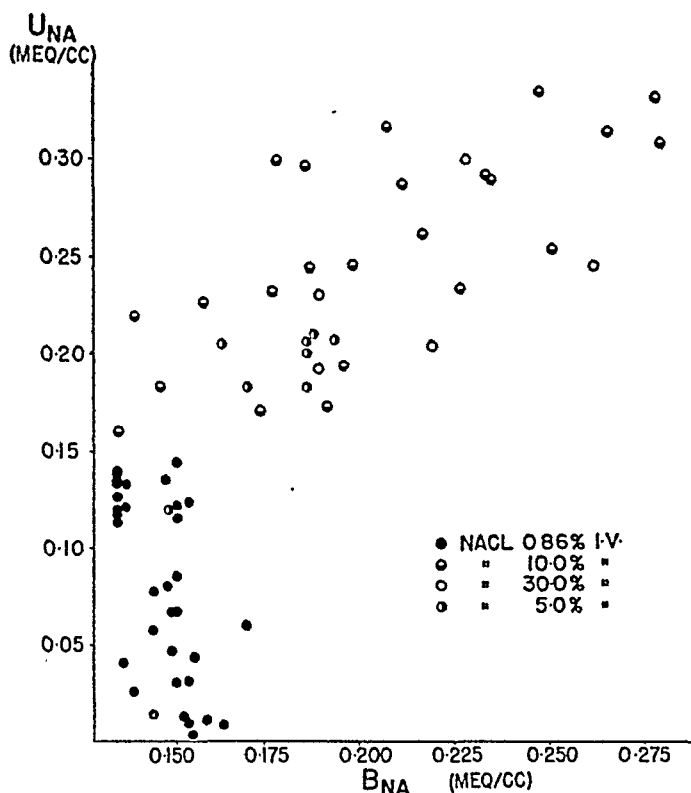


Fig. 4. RELATION OF SODIUM CONCENTRATIONS in urine and plasma under varying conditions of sodium load.

The maximal rate of sodium excretion was not proportional to the sodium balance, the extracellular sodium or the sodium space, either in the same animal or in comparisons between animals. Low rates of sodium excretion were often found in the presence of a markedly positive sodium balance, suggesting that the increase in this and related quantities (extracellular sodium mass and sodium space) rather than constituting a stimulus to sodium excretion was instead the consequence of failure by the animal to respond to sodium loading by an adequate increase in renal excretion. Comparison of these factors in animals infused at comparable rates of sodium and water administration (fig. 5) showed that the animal which developed the smaller sodium excretory rate manifested the larger increment in sodium balance, extracellular sodium mass and sodium space.

*Water Balance.* Administration of hypertonic solutions was followed almost

FIG. 5

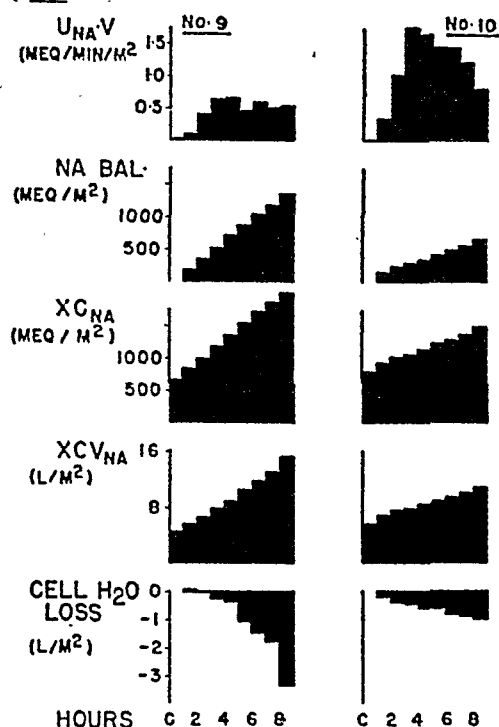


FIG. 6

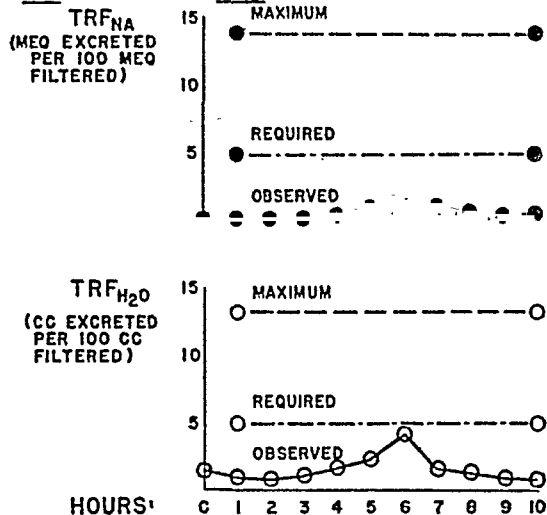


Fig. 5. INVERSE RELATION of sodium excretion to sodium balance and associated factors, where:  $U_{Na} \cdot V$  = Sodium excretion;  $Na\ Bal.$  = Net sodium exchange;  $XC_{Na}$  = Extracellular sodium mass;  $XCV_{Na}$  = Extracellular sodium space;  $CELL\ H_2O\ loss$  = Cumulative cellular dehydration.

Fig. 6. COMPARISON OF TUBULAR rejection fractions observed during infusion of 0.86% NaCl at the rate of 0.6 mEq/min/m<sup>2</sup> with the rejection fractions which would have been required for maintenance of sodium and water balance. Indicated maximum rejection fractions were measured in other animals infused with the same sodium concentration at higher rates.

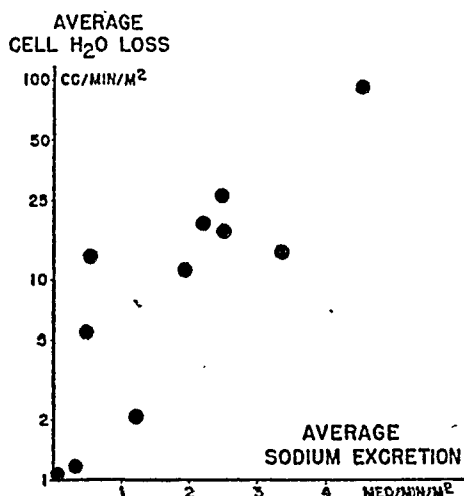


Fig. 7. RELATION OF AVERAGE RATE of sodium excretion to average rate of cellular dehydration during administration of NaCl solutions.

uniformly by a negative water balance, while isotonic solutions produced a positive balance (table 1, column 7). No correlations were established between the rate of sodium excretion and the direction or magnitude of the net water gain or loss.

**Hematocrit.** Serial hematocrit values were determined on 6 animals as an index of blood volume change. Administration of the test solution was followed by a fall in

hematocrit value, succeeded in some instances by a secondary rise (table 1, animals 7, 9, 10). In all but one case the minimum level coincided with or followed the period of maximum sodium output. Beyond this no correlation between the two factors was established.

**Cellular Dehydration and Osmotic Balance.** In the majority of animals observed for periods greater than 3 hours the rise in sodium excretory rate was succeeded by a decline in output despite an increasing distortion of the sodium balance and its associated factors (table 1, animals 7, 8, 9, 10, 12). The secondary decline in sodium output did not appear to be a consequence of the exhaustion of the animal's mechanism for sodium excretion, for it occurred even at low sodium injection rates during

which blood pressure, filtration rate and plasma flow were maintained or increased, and the maximum rate of excretion obtained was well below the maximum capacity for excretion as judged by the response of other animals to higher rates of sodium infusion at isotonic concentrations (fig. 6). The pattern of this response suggested the possibility that the initial rise in sodium excretion was due to a disturbance of osmotic equilibrium, while the secondary fall reflected a subsequent return toward equilibrium despite continued infusion.

Increased sodium output has been demonstrated to follow the subjection of osmoreceptor cells to concentrated solutions of sodium chloride injected into the

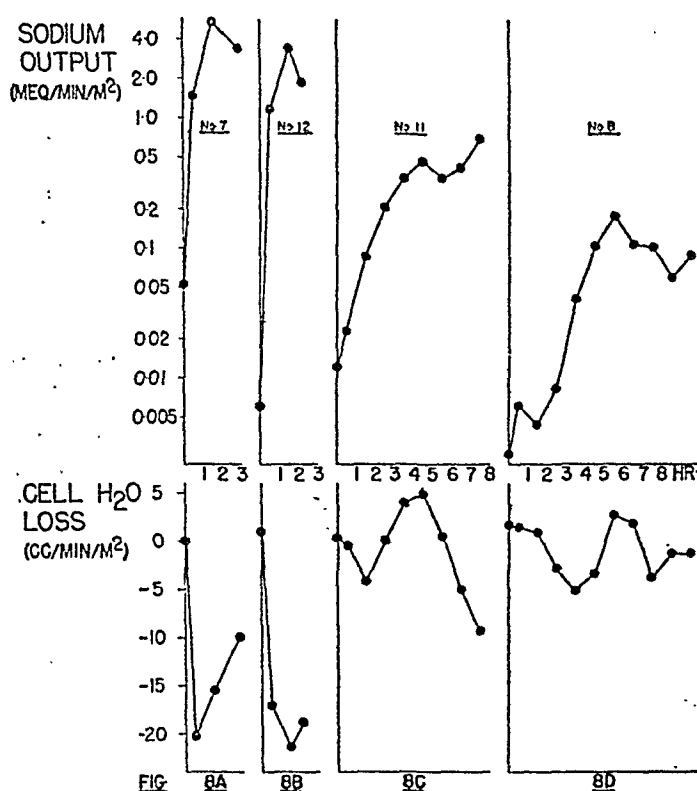


Fig. 8. RELATION OF SODIUM OUTPUT to serial changes in cellular dehydration rate during continuous infusions of NaCl solutions.

carotid artery (6). Since long-term (40-minute) infusions of dextrose of the same osmolarity were without effect, it appears likely that the stimulus to osmoreceptor cells depends on exposure to hypertonic concentrations of a non-permeating solute, resulting in osmotic pressure differences productive of cellular dehydration. Under these circumstances the rate of cell water loss becomes a measure of the osmotic pressure difference on the two sides of the cell membrane.

In the present experiments a mechanism for cellular dehydration was obvious in animals to which hypertonic solutions were administered. In all animals, however, an endogenous source of dehydration was presented, regardless of infusion concentra-

tion, by the continued secretion of a urine with a lower sodium concentration than plasma for varying periods after the start of the infusion (table 1, columns 10, 12). The operation of these factors was evidenced by the finding that the net change in sodium space was uniformly greater than the net water exchange, and could have been accomplished only by withdrawal of water from cells, barring a change in permeability toward sodium (table 1, columns 7, 19).

When the rapidity of cellular dehydration was computed by dividing the cell water change at the end of each period by the time over which it had occurred it was found that the average rates of sodium excretion and cellular dehydration were correlated ( $r = 0.788$ ; fig. 7). A causal relationship was suggested by the temporal association of the two factors (fig. 8). Cellular dehydration was most intense in the early phases of the experiment. In 7 animals the maximum rate of cellular dehydration preceded the maximum rise in sodium excretion by an interval which ranged from 20 minutes to 3 hours. As sodium output increased the rate of cell water loss tended to fall. In some instances the magnitude of this reversal was sufficient not only to prevent further dehydration but to return water to cells (fig. 8 c & d).

In those animals in which the reversion of the rate of cell water loss was marked, a reduction in sodium output followed, regardless of the net sodium balance (fig. 8). When the infusion was continued for a sufficiently long period, this fall in sodium output was followed by a new cycle of increased cellular dehydration rate followed by a secondary rise in sodium output (fig. 8 c, 8 d).

Interpreting these results, it would seem that changes in sodium excretion are invoked by differences between the extracellular and intracellular osmotic pressures and persist only as long as such differences are maintained.

#### SUMMARY

The alterations in renal function which followed the administration of sodium chloride solutions were measured in a series of normal dogs. The resultant rise in sodium excretion was found due almost entirely to a decrease in its tubular reabsorption, and was accompanied by a correlated decrease in the reabsorption of water. Renal plasma flow was uniformly increased. No consistent change in glomerular filtration rate was demonstrated.

Increased sodium intake appeared to influence sodium excretion primarily by establishing an osmotic pressure difference between extra- and intracellular fluids, as estimated by the rate of cell water loss. Resolution of such osmotic pressure differences, as judged by a decline in the cellular dehydration rate, was followed by a fall in sodium excretion regardless of the magnitude of the plasma sodium concentration, sodium balance, extracellular sodium mass or sodium space. The data suggest that the major determinant in sodium regulation is not the preservation of sodium balance but the maintenance of osmotic homeostasis.

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# ALTERATION OF NEURON EXCITABILITY BY RETROGRADE DEGENERATION<sup>1</sup>

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THE definition and understanding of the altered physiological activity of the chromatolysed, degenerating and diseased nerve cell are fundamental problems in neurology. It is essential that the well-known pathological pictures of nerve cells be correlated with their functional concomitants before the symptomatology and recovery processes from neurological disorders, acute and chronic, can be understood. In addition, the problem of the nature and function of cytological structures depends to a great extent on the use of experimental alterations of cellular elements as a tool in linking anatomical entities with specific functions.

It is only natural that chromatolysis, the experimental reaction most easily controlled, should be the first cell alteration to be subjected to this analysis. Acheson, Lee and Morison (1) cut the phrenic nerve and noted a change in the activity of the motor cells, during the respiratory cycle, which roughly paralleled the usual period of chromatolysis and was for the most part a deficiency in cell firing. The loss of the proprioceptive or two-neuron reflex during the chromatolytic cycle of the motor neurons has been described (2) and it has been demonstrated that chromatolytic dorsal root ganglion cells show no loss of conductile properties (3) and that the antidromic cell potential is decreased during the chromatolytic period (4). These facts indicate that the interference of cell function occurs at the cell body level and involves neither the sensory side of the reflex arc nor the synaptic apparatus.

The theory that the physiological effect of chromatolysis is on one general threshold of irritability of the cell rather than on the mechanisms of specific reflex pathways, as the evidence cited above indicates, leads to interesting consequences. One is that any reflex such as the tibial-peroneal reflex of Bernhard (5) will serve as a test for the general phenomenon. Any result from inspection of the effects of chromatolysis on this reflex which differ from those mentioned above would render the theory untenable. Strengthening of the theory, however, which would result from the demonstration of the predicted results in the instance of such an indirect reflex would require an inspection of the current theory of neuron activation.

A further consequence of the theory that the threshold of excitability is responsive to the axon reaction is that chromatolysed nuclei would show certain predictable changes in the relative numbers of cells falling into the subliminal fringe upon the application of single afferent stimuli. As the threshold of the neurons of the nucleus declines, the fraction of cells excited subliminally would increase with respect to those raised above threshold. However, as the rate of decreased excitability wanes, the original fraction value might well be re-established.

## METHODS AND RESULTS

Cats and rabbits were used in the experiments. Sectioning of the combined tibial and peroneal nerve on the right side at mid-thigh level was done with aseptic

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precautions under nembutal anesthesia. After a suitable recovery period, anesthesia was again produced with dial or nembutal and the reflex activity tested on both the affected right side and on the normal left side which served as control. A three-channel sweep-synchronized stimulator, condenser coupled amplifier, and cathode-ray oscillograph were used for the physiological testing. The spinal cords were fixed in formalin, sectioned and stained with cresyl-violet and erythrosin for study of chromatolysis.

TABLE 1. EFFECTS OF AXON SECTION ON TIBIAL-PERONEAL REFLEX OF CATS

CAT NO.	DAYS DE-GENERATED	INCREASED LATENCY, MSEC.	FORM AND SIZE	CHROMATOLYSIS TYPE
1	2	0.3	Normal form, size	1
2	3	0.5	Normal form, reduced	2
3	4	0	Simplified, reduced	2
4	5	1.2	" "	2
5	6	0.3	" "	2
6	7	0	" "	2
7	8	1.8	" "	2
8	8	0	" "	2
9	9	0.2	Normal form, size	2
10	9	0	Simplified, reduced	*
11	9	2.0	" "	*
12	10	0.3	" "	2
13	12	1.1	Normal form, size	2
14	12		Absent	*
15	13		"	2
16	13	3.6	Simplified, reduced	*
17	13		Absent	2
18	14		"	*
19	15		"	2
20	18	3.0	Simplified, reduced	2
21	27	2.8	" "	3
22	27	2.1	" "	3
23	34	0.5	Simplified, normal size	3
24	35	1.5	Simplified, reduced	2
25	102	4.8	" "	*

\* No Nissl studies made.

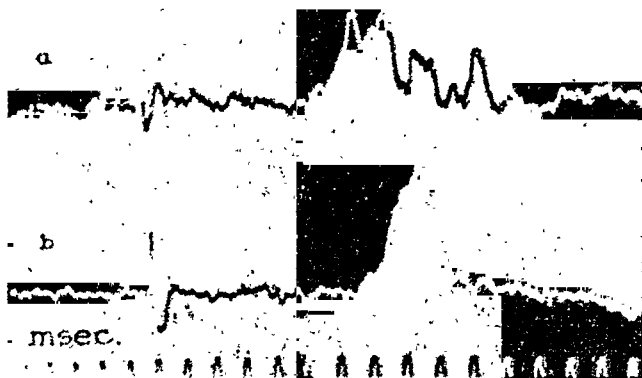
A series of twenty-five cats was studied (table 1). The days of retrograde degeneration varied from 2 to 102 days, with most careful coverage on the first two weeks. The changes noticed in this series were of four types. There was an increased latency of the response of the peroneal nerve following tibial stimulation. This was seen in every animal from 9 days on as well as in 4 of 8 which were tested earlier. Only one animal, at two days, showed the reverse trend; three others, at 3, 6 and 8 days, showed no change. In the later experiments, significant delays were found, ranging up to 3.6 msec. Interpretation of the early half of the series is complicated by the fact that the latency difference between the two sides is apparently of the order of magnitude of the variability of the preparation. Attempts were made to

insure equal conduction distance on the left (normal) and right (chromatolysed) side but these were not always successful. The errors introduced by this factor appear to range up to 0.5 msec. No interpretation is attempted on the 2-day animals, where a decreased latency appeared.

The second effect shown in this series was an abolition of the reflex completely during the height of the degenerative cycle. This occurred in 5 animals, at 12, 12, 14, 15 and 26 days. All animals of this period do not show this loss, but those which do not show the complete loss show very extended latencies.

The third effect noted is the simplification of the form of the reflex curve. Figure 1 shows a typical example in which the normal reflex pattern—usually a slowly decreasing multimodal curve exhibiting three, four, or even five definite peaks of activity—was altered on the chromatolysed side to produce a much simpler curve. The dispersal of the normal activity strongly indicates repetitive firing of the motor neurons. We are led, thus, to interpret the simplification as shown in figure 1 as an indication of the shortening of the train of impulses generated by the individual

Fig. 1. ALTERATION OF REFLEX RETURN on peroneal nerve of cat in response to tibial stimulation on normal (a) and chromatolysed (b) sides. The duration of the retrograde degeneration was 35 days.



motor cell. Through some mechanism which is not obvious, synchrony of the initial discharge sometimes characterizes the altered response with the curious result that the amplitude of the response as measured by peak voltage is actually increased (see below).

A fourth consequence of the chromatolysis was found to be a decrease in the total reflex outflow. Because of the tendency for synchrony as mentioned above, the height of the reflex record on the affected side was occasionally increased, but even in such cases a decreased return was obvious. Because of the dispersion of this particular reflex, peak voltage is a poor measure of the actual response. We have used, instead, the area under the potential curve as a measurement of the total activity. Where strong synchrony and no repetitive firing are the rule, as in the proprioceptive reflex, close correlation must exist between the height of the response and the number of units active. With monophasic recording, the same correlation would obtain between the number of conducted impulses and the areal summation of the curve. We may conclude, thus, that the activity as measured in impulses is decreased on the chromatolysed side.

In summary, it is to be seen that the four functional alterations observable in the chromatolysed motor nuclei of these cats are obviously the result of a dysfunction

which, acting to decrease excitability, makes the reflex return to a supramaximal (for A fibers) shock to the tibial nerve later, simpler, smaller and occasionally absent.

Study of stained sections of the spinal cords showed chromatolysis in the earliest member of the series (two days) and apparent in all in which Nissl studies were made. The classification proposed by Campbell and Novick (6) of the stages of axon reaction was used to record the degree of chromatolysis seen in the sections. However, no quantitative expression was attempted because it was not feasible with the material on hand to attempt to circumscribe the nuclei involved. The numerical stages indicated in table 1 represent the most prominent class of altered cells in the sections sampled. These findings agree essentially with those of Barr and Hamilton (7), who have studied the time course of the axon reaction in the cat using similar categories.

TABLE 2. CONDITIONING EFFECT OF TWO STIMULI IN RABBITS

RABBIT NO.	DAYS OF DEGENERATION	AVERAGE AREA OF TEST VOLLEY EXPERIMENTAL NORMAL	INCREASED LATENCY MSEC.	MAX. AREA OF CONDITIONED RESPONSE AS % OF 2X TEST RESPONSE	
				Normal	Experimental
1	3	33%		84	120
2	3	51		79	101
3	5	12	.07	100	165
4	5	40	.55	95	135
5	5	103	.60	95	155
6	5	11	.21	85	155
7	8	21	.54	65	98
8	8	51	.50	80	80
9	8	13	.33	75	155
10	11	24	.50	80	70
11	12	17	.74	79	79
12	12	64	.62	100	79
13	15	78	-.2	76	58
14	16	61	1.55	109	83

Some mention should be made of the fact that the classifications mentioned above do not serve adequately for study of the recovering cell. It would appear that the later stages of the chromatolytic cycle do not merely retrace the rising phase. Thus, the designation of the cells of cat 24 as being in the second stage is provisional only.

In similar experiments in a series of 14 rabbits, in addition to the observation of the alteration of the simple reflex, a systematic study was made of the conditioning curves obtained by two shock stimulation. Table 2 shows the data obtained. Retrograde degeneration was studied from 3 to 16 days' duration. In none of the rabbits was there a complete obliteration of the reflex on the degenerated side as had been seen in some of the cats. The decrease in the reflex noted in the former series was similarly revealed in the rabbits (column 3, table 2). With the exception of one animal, there was a considerable decrease in the area under the potential curve of the returned reflex on the affected side. Latency was increased from 0.3 to 1.5 msec., with but one exception Figure 2 illustrates a typical experiment and the simplification of the pattern on the right side conforms with the rule.

The analysis of the conditioning curves of the rabbit series reveals an additional

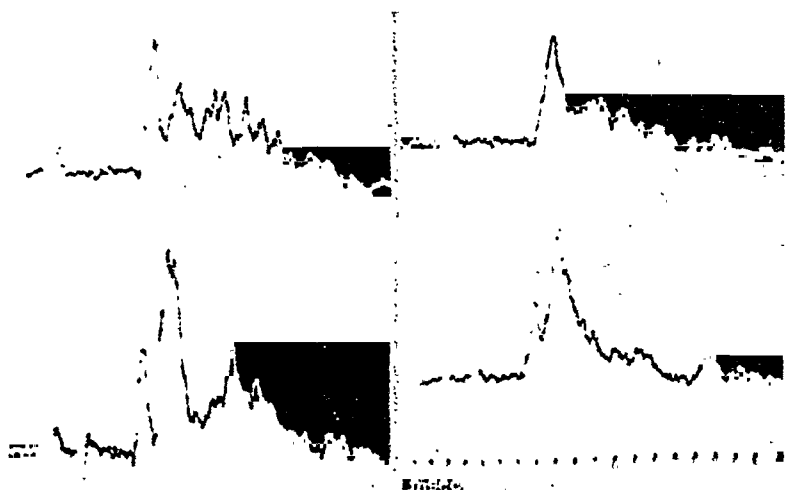


Fig. 2. ALTERATION OF REFLEX RETURN on peroneal nerve of rabbit in response to single and double stimuli. Left side of figure shows normal side, right shows experimental side. The duration of degeneration was 3 days.

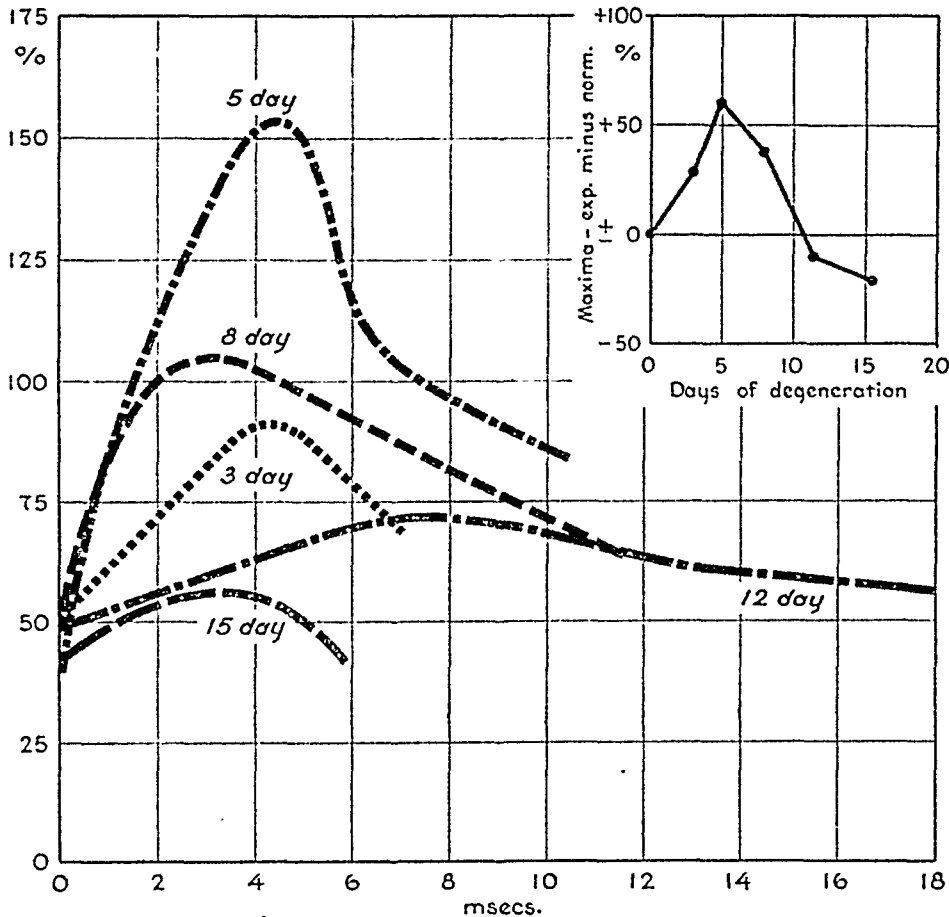


Fig. 3. CONDITIONING CURVE OF REPRESENTATIVE RABBITS SHOWING conditioning curves of degenerated side in percentage of twice the area under the test potential curve. *Insert.* Comparison of degenerated side with normal plotted according to durations of retrograde degeneration.

sign of threshold involvement on the part of the final common pathway neurons. The second of two supramaximal shocks produces a reflex on the experimental side which greatly differs from that on the control side, (fig. 3). This difference varies

with the duration of the axon section. In column 5 of table 2 the maximal electrical activity attained by two successive supramaximal shocks is listed as a percentage of twice the activity of the test shock. The differences are obtained by subtracting the figure for the normal side from that of the degenerated side. These figures are averaged for the various days of degeneration and plotted as a function of time in figure 3 (insert). Even though the relative percentage values of the abnormal side may exceed its control at short degenerative intervals the absolute values of total electrical activity are always much less on the experimental side.

The curve of conditioned response to two supramaximal shocks, (fig. 3), may be taken as a profile of the subliminal fringe excitability, with the added factor, especially in the very short intervals, of the refractory periods of the various portions of the reflex arc. The maximum of the curve, which is to be found at the interval of several milliseconds, is probably only insignificantly more correlated with any other factor than the excitability of the subliminal fringe. Thus we may interpret the results shown in column 6, table 2 and figure 3 as indicating a large (percentagewise) subliminal fringe in the early stages of this series, declining to below normal in the 16-day group. Associated as this is with a decreased reflex return to a single shock, this then may be interpreted as increased threshold of the member cells of the nucleus stimulated. With the slight increase of threshold in the early series, a large percentage of those failing to respond to the first stimulus were available to the second. With the more profound decrease in excitability, relatively fewer were left with a residuum of activity after the first stimulus sufficient to help achieve effective threshold as a result of the second.

#### DISCUSSION

The axon reaction is known by several morphological criteria. Cells with axons severed show a cycle of degenerative changes in the cell body. Best known of these are the fragmentation and the pulverising of the Nissl granules and the alterations in the position of the cell nucleus. Increased volume of the cell, altered enzyme concentrations and nuclear cap formation are also known. It cannot be assumed that all or any of these phenomena are directly correlated with the altered physiology. For the present, however, we must consider the mechanics of neuronal excitation in the light of current models of the origin of the conducted impulse and some refinement of these ideas may be devised from the data offered above.

The simplest model of the reflex arc will not suffice here. Simple all-or-none transmission over the hypothetical synapse would be insufficient to explain the graded nature of the changes of response associated with retrograde degeneration of the nerve cell. A local graded response at the level of the neuron affected must be assumed and two current models, each the product of many minds, may be examined. The first is based upon a consideration of the external electrical fields produced by resting and active neurons, and on the effects upon the cells of polarizing currents. In a paper discussing galvanotaxis in the crayfish, Loeb and Maxwell (8) presented a diagram from which nearly all of the more recent refinements of the theory may immediately be deduced but a thorough exposition is lacking in the accompanying text. Forbes (9), Barron and Matthews (10), Gerard and Libet (11) and Gesell (12)

have developed the model; the last-named author contributing a discussion of the idea that the conducted impulse is generated at the axon hillock. Briefly, a cell is postulated which is organized in such a way that its metabolic activity is reflected in an electrical bipolar field involving current flow both within and external to the cell. The field is assumed to be oriented with the axis of the neuron so that in steady states a certain current density exists at the membrane of the axon hillock, which serves as a pulse-signal generator in that the conducted impulses arise at this point and correspond, in frequency, to the flux. An alteration of the external field, as induced by the explosive negativity of the boutons, or from any other phenomenon will effect the current density at the axon hillock and transmit signal by modulating the frequency of the generation of conducted impulses. It should be remarked here that the ventral horn cells would seem to exist in a steady state of zero frequency of the conducted impulses, at least in the experimental situation described in the paper. The current alternative to the bipolar model discussed above is an evolutionary product of the Lucas dictum (13) and has been best described in the analysis of Lorente de N6 (14). In this scheme of neuron activity, signal is transmitted by conduction of active states from the boutons or other perineuronal endings of specific telaxons across a physiologically defined gap, the synapse, to excite conducted waves of depolarization on the cell membrane of the body and/or dendrites of the secondary neuron. Graded correlation of afferent and efferent activity may be achieved by the operation of local responses, a function of the secondary cell, which by varying with the state of the cell metabolism (hence polarity of membranes) would show altered spatial and temporal summation. The ability of the local responses to achieve the magnitude necessary for the local generation of a conducted impulse would be graded, under experimental conditions such as considered in this paper, to give results such as actually obtain. Between these two models we may not conclusively choose on the basis of the present data. In the absence of a *tertium quid*, the only slight contribution possible is to remark that the morphologic variants with this cycle of altered response affect the internal structure of the cell and not at least in a way to be observed at the present time, the cell membrane. From this some slight preference to the bipolar model may be expressed.

The cytological alteration studied here is 'unphysiological' in the sense that axon destruction is rarely if ever of occurrence in the normal animal. There is no reason to think that this particular type of degeneration plays a part in the economy of the animal. Yet there is a possibility, supported by a small but insistent literature, that cytological alteration similar to this type of chromatolysis may result from over-activity of nerve cells. Barr and Hamilton (7) found slight chromatolysis in over 10 per cent of the motor cells of their control spinal cords. If this should prove well-founded, the importance of these changes in the irritability of the cell might well have to be reckoned as having some function in the integrative process.

#### SUMMARY

A study was made of the tibial-peroneal reflex of cats and rabbits following intervals of retrograde degeneration of the primary motor neurones of the peroneal nerve of 1 to 102 days.

Chromatolysis is associated with an alteration of the reflex activity of the spinal motor nuclei. The tibial-peroneal reflex, during the cycle of retrograde degeneration shows *a*) an increase in latency, *b*) an occasional complete cessation of reflex transmission, *c*) simplification of pattern of reflex when present, and a decrease in the activity of the involved neurons as measured by the area under the curve of the efferent potential. Conditioning studies indicate that there is a relative increase, during the rising phase of the cycle, of the subliminal fringe which is a measure of the increased threshold of the cells.

The findings are discussed in the light of current models of the neuron.

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# EFFECT OF 2-METHYL NAPHTHOQUINONE ON THE ACTION POTENTIAL OF NERVE AND MUSCLE

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THE effect on the action potential of nerve and muscle of a potent and probably specific inhibitor (1-3) (2-methyl naphthoquinone) of choline acetylase was investigated to ascertain whether or not it impairs the function of nerve and muscle. Should impaired function be exhibited, and, assuming that disturbances in acetylcholine synthesis under the circumstances is the only biochemical action of the naphthoquinone, it might be inferred that acetylcholine is essential to the maintenance of proper neuro-muscular function.

## METHOD

Action potential measurements were made on *a*) isolated frog organs and *b*) organs of mouse and rat *in situ*. Over 20 preparations were used in each group.

*A. Isolated Frog Organs.* Either the sciatic nerve or the sciatic nerve together with the attached gastrocnemius muscle were suspended in frog Ringer's solution at room temperature (23° C.). During the entire experiments a gas mixture containing 5 per cent carbon dioxide and 95 per cent oxygen was bubbled through the bathing fluid.

*B. Organs in Situ.* The spinal cords of mouse and rat were destroyed up to the midthoracic region under light ether anesthesia. A few hours afterwards the animal and its leg at the knee joint were firmly fixed to an animal board.

*Action Potential Measurements.* Stimulating bakelite insulated silver wire electrodes, 4 mm. apart, were placed under the nerve. The same type of recording electrodes were placed under the nerve (2 cm. apart) and in the muscle (one in the tendon of the gastrocnemius muscle and the other in the upper half of the muscle). Stimuli with a repetition rate of 11 pulses per second and of 'supramaximal' intensity were delivered every 10 minutes for 30 seconds. Supramaximal intensity was maintained by increasing the intensity of the stimulus wherever the threshold increased. The action potentials of the nerve and muscle (either diphasic or monophasic) were observed by a cathode ray oscilloscope. The sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and action potentials were superimposed on the screen of the cathode ray tube. Either single or superimposed action potentials were photographically recorded.

*2-Methyl Naphthoquinone.* After the action potential remained constant for two to three series of stimulations the effect of the naphthoquinone was tested by the following method: *a*) The isolated organs were immersed during the 10-minute rest periods into a Ringer's solution containing the naphthoquinone in concentrations of  $1 \times 10^{-5}M$ ; *b*) the animals were injected with the naphthoquinone intraperitoneally in amounts computed to give a final body concentration of  $1 \times 10^{-5}M$ .

*Direct Stimulation of Muscle.* The muscle was directly stimulated with a current having a repetition rate of 5 pulses per second and being of 'supramaximal' intensity. This direct stimulation was delivered before and immediately after repetitive indirect stimulation. The muscle contraction was recorded by a kymograph through an isotonic lever attached to the end of the tendon of the gastrocnemius muscle.

*Controls.* Isolated preparations and living animals treated and stimulated as described above (except that they were not treated with the naphthoquinone) served as controls.

## RESULTS

*A, 1. Isolated Nerve Preparation.* The nerve was stimulated for 30 seconds at 10-minute intervals. The threshold, the amplitude of the first action potential, as well as the conduction velocity, of the control nerves remained unaltered for several

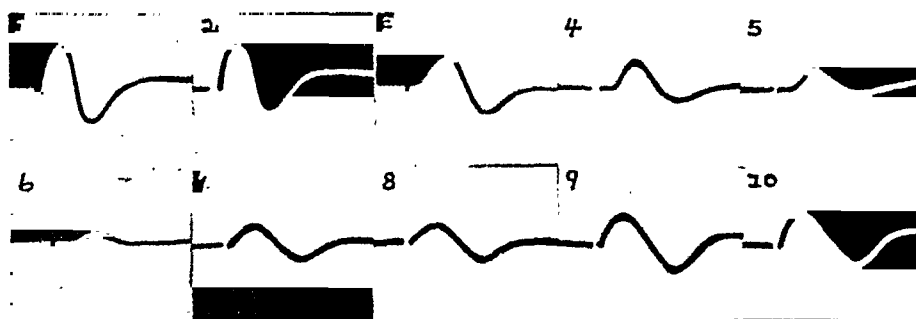


Fig. 1. ACTION POTENTIAL RECORDS OF ISOLATED NERVE (stimulation with 11 pulses per sec.). 1) Action potential taken at the beginning of 30-sec. stimulation period, nerve immersed in Ringer's solution; 2) action potential taken at the end of the 30-sec. stimulation period; 3) same as 1 taken after 1-hour immersion in the solution of the naphthoquinone; 4) same as 2 taken after 1-hour immersion in the solution of the naphthoquinone; 5) same as 1 taken after 1½-hour immersion in the solution of the naphthoquinone; 6) same as 2 taken after 1½-hour immersion in the solution of the naphthoquinone; 7) same as 1 taken after 20-min. washing with Ringer's solution; 8) same as 2 taken after 20-min. washing with Ringer's solution; 9) same as 1 taken after 40 min. washing with Ringer's solution; 10) same as 2 taken after 40-min. washing with Ringer's solution.

hours. The amplitude of the action potential decreased on the average 5 per cent during each 30-second stimulation period. Complete recovery occurred during the 10-minute rest periods.

The conduction velocity and the amplitude of the action potential of the nerve decreased and the threshold increased when immersed in solutions of the naphthoquinone. An immersion of 45 minutes or more was required before the appearance of the first changes in nerve function. The amplitude of the action potential decreased on the average 30 per cent during each 30-second stimulation period. Recovery did not occur during the 10-minute rest periods in the naphthoquinone solution (fig. 1). Finally, the action potential disappeared almost completely. Prolonged washing with Ringer's solution reversed the effect of the naphthoquinone. Recovery of the conduction velocity preceded the recovery of the amplitude of the action potential.

*2. Isolated Nerve-Muscle Preparation.* The threshold and the interval of time elapsing between the stimulating shock and the appearance of the muscle action potential, with nerve-muscle preparations immersed only in Ringer's solution, remained unaltered for at least one hour. The amplitude of the muscle action potential usually increased during the first few seconds of stimulation and did not decrease during the

30-second stimulation period as compared to the first action potential of this stimulation period. The amplitude of the first action potential of each 30-second stimulation period remained unaltered for at least half an hour and decreased on the average by 20 per cent at the end of one hour.

Immersion of nerve-muscle preparations in solutions of the naphthoquinone caused within a few minutes an increase in the latency and the threshold, and a decrease in the amplitude of the action potential. The amplitude of the action potential decreased on the average 50 per cent during each 30-second stimulation period. Recovery did not occur during the 10-minute rest periods in the naphthoquinone

Fig. 2. ACTION POTENTIAL RECORDS OF ISOLATED GASTROCNEMIUS MUSCLE during stimulation of the sciatic nerve (stimulation with 11 pulses per sec.) 1) Action potential taken at the beginning of the 30-sec. stimulation period before immersion in the solution of the naphthoquinone; 2) action potential taken at the end of the 30-sec. stimulation period; 3) same as 1 taken after 10-min. immersion in the solution of naphthoquinone; 4) same as 2 taken after 10-min. immersion; 5) same as 1 taken after 30-min. immersion; 6) same as 2 taken after 30 min. immersion.

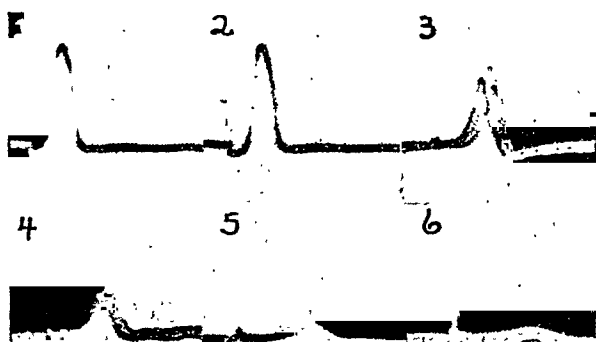
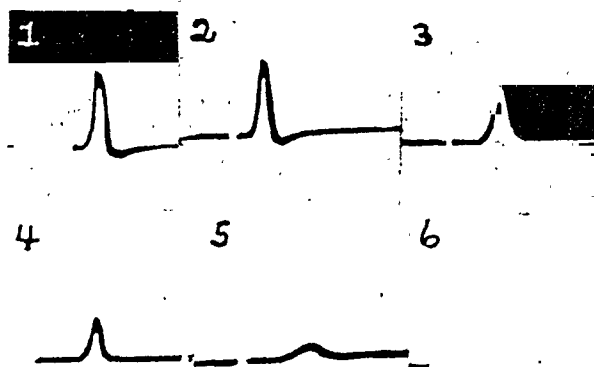


Fig. 3. ACTION POTENTIAL RECORDS OF GASTROCNEMIUS MUSCLE of rat *in situ* during indirect stimulation (stimulation with 11 pulses per sec.) 1) Action potential taken at the beginning of a 30-sec. stimulation period before injection of the naphthoquinone; 2) action potential taken at the end of the 30-sec. stimulation period; 3) same as 1 taken 10 min. after injection of naphthoquinone; 4) same as 2 taken 10 min. after injection; 5) same as 1 taken 30 min. after injection; 6) same as 2 taken 30 min. after injection.

solution. Within 30 minutes the amplitude of the first action potential of the 30-second stimulation period averaged 25 per cent of the amplitude of the corresponding first action potential before immersion in the naphthoquinone solution (fig. 2).

*B, 1. Indirect Stimulation of Muscle in Situ.* The threshold, the amplitude of muscle action potential, as well as the conduction velocity, remained unaltered in the control animals during indirect stimulation for several hours. The amplitude of the action potential decreased on the average 10 per cent during each 30-second stimulation period. Complete recovery occurred during the 10-minute rest periods.

In animals injected with the naphthoquinone the amplitude of the muscle action potential decreased and the latency and the threshold increased during indirect stimulation. The amplitude of the action potential decreased during each 30-second stimulation period on the average by 45 per cent. Recovery did not occur during the 10-minute rest periods. The action potential finally dropped to zero (fig. 3).

2. *Direct Stimulation of Muscle in Situ.* In the control animals the magnitude of contraction of the gastrocnemius muscle remained unchanged during repetitive indirect stimulation. The magnitude of contraction on repetitive direct stimulation before and after indirect stimulation, also remained the same.

In injected rats the contraction of the gastrocnemius muscle on repetitive indirect stimulation decreased in a degree similar to that of the action potential. The magnitude of muscle contraction on repetitive direct stimulation before and after indirect stimulation was approximately the same (fig. 4).

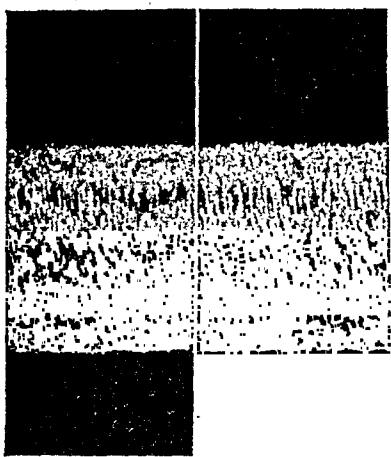


Fig. 4. KYMOGRAPH RECORDS OF THE GASTROCNEMIUS MUSCLE during direct stimulation (stimulation with 5 pulses per sec., rat). *Left*, record taken before indirect stimulation in a rat injected with the naphthoquinone. *Right*, record taken immediately after indirect stimulation.

#### DISCUSSION

✓ The experiments indicate that 2-methyl naphthoquinone decreases the conduction velocity and the amplitude of action potential of nerve and increases the threshold. The contractile mechanism in the muscle is, however, not impaired in the presence of 2-methyl naphthoquinone in a concentration of approximately  $1 \times 10^{-5}M$  since the muscle contraction on repetitive direct stimulation did not change significantly.

These results agree with those reported from this laboratory (4) showing that inhibitors of choline acetylase decrease the response of striated muscle to indirect stimulation as measured by myography. This decrease simulates a curare effect but is induced by a different mechanism. Curare interferes at the receptors of the effector cells with acetylcholine, whereas the naphthoquinone is a potent inhibitor of choline acetylase (1-3) and does not interfere, in the concentrations used, with acetylcholine at the receptors of the effector cells (1).

The decreased nerve and muscle function found in the presence of naphthoquinone in concentrations of  $1 \times 10^{-5}M$  was probably due to its effect in decreasing acetylcholine synthesis, since the concentrations required to inhibit the activity of enzymes other than choline acetylase—cholinesterase (1), urease (5), catalase (6), papain (7), and enzymes involved in aerobic and anaerobic lactic acid formation (8)—are greater than  $1 \times 10^{-5}M$ . Other inhibitors of choline acetylase—monoiodoacetate, iodoacetate (9-11), toxin of *Clostridium botulinum* (12, 13), alloxan (4, 14-16),  $\alpha$ - and  $\beta$ -naphthol (4, 14-16)—were also found to decrease the action potential (11, 4, 16). The results with many of these agents cannot, however, be interpreted as being ef-

fect only through inhibition of acetylcholine synthesis, e.g. iodoacetate and iodoacetamide inhibit, in the concentrations used, the metabolism of triosephosphate (17) causing a decrease of regeneration of energy-rich phosphate bonds. Therefore, iodoacetate and iodoacetamide probably act through many mechanisms besides inhibition of acetylcholine synthesis.

Acetylcholine probably acts at more than one point. It is, however, likely, that the anoxic depolarization of nerve is dependent on the presence of acetylcholine. This assumption may be made since *a*) anoxia induces depolarization of nerve (11) without an inhibition of the activity of choline acetylase (10); *b*) iodoacetate prevents anoxic depolarization (11) and inhibits acetylcholine synthesis (9, 10); and *c*) most of the other processes known that could be considered as the cause of anoxic depolarization (e.g. inhibition of glycolysis resulting in interrupted regeneration of adenosinetriphosphate) are inhibited in the nerve by both iodoacetate (17) and anoxia (11).

#### SUMMARY

The effect of 2-methyl naphthoquinone on the function of nerve and muscle was investigated *in vitro* and *in vivo*. In the presence of low concentrations of the naphthoquinone the latency and the threshold increased and the amplitude of the action potential of nerve and muscle decreased. The muscle function on direct stimulation was not significantly impaired. Since the naphthoquinone, in the concentrations used, is known to inhibit only the activity of choline acetylase, the dysfunction observed suggests that acetylcholine is necessary to maintain an optimal function of nerve.

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# MUSCLE RECOVERY AFTER NERVE SECTION AND SUTURE

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**T**HAT partially denervated skeletal muscle makes some spontaneous recovery, improving in weight and strength, was shown in three separate but almost simultaneous reports by van Harreveld (1), Hines, Wehrmacher and Thompson (2) and Weiss and Edds (3). The general experimental procedure used by these three groups was to remove a portion of the spinal roots supplying innervation to a skeletal muscle. There was then an early loss of weight and strength of the muscle followed by some spontaneous improvement. This improvement was proved not due to regrowth of the sectioned nerve roots. Rather it seemed to depend upon two factors: *a*) hypertrophy of the remaining innervated muscle and *b*) an increase in the number of innervated muscle fibers through adoption of some of the denervated muscle fibers by axone branches from the intact motor nerves. It seemed as if this second factor was the most important in the recovery.

It has long been suggested that by inducing increased branching of peripheral motor axones an improvement in partially denervated muscle could be obtained. Claims for such induced branching with good clinical results, have been made by Feiss (4) and Dogliotti (5). In these instances axone branching was produced by nerve section and suture or by nerve crush.

A much more extensive series of attempts to mobilize the residual nerve supply for reinnervation of partially denervated muscle was reported by Billig, van Harreveld and Wiersma (6). Their results did not show the spectacular success of Feiss (4) and of Dogliotti (5) but they concluded that nerve crushing (neurotripsy) by either an open or closed method did, in many instances, result in muscle improvement. This was particularly the case if the muscle had a poor innervation before neurotripsy was done. On the other hand, many of the better innervated muscles suffered a loss in size and function as a result of the neurotripsy procedure.

The question arises whether such a deliberate nerve section or crush will really enhance the tendency that intact fibers have for adoption of denervated muscle fibers. A recent report by Fredrick and Kossman (7) indicates that for the partially denervated anterior tibial muscle of the dog closed manual neurotripsy results in no increase of muscle weight and strength over that which will occur spontaneously.

In the experiments described here the question was posed as to whether the extent of recovery in a reversibly denervated muscle could be enhanced by neurotripsy. Rats were used as the experimental animal. A nerve lesion was made by cutting the sciatic nerve at the level of the greater trochanter and repair made by immediate end to end suture of the nerve. The nerve was cut with a fine scissors rather than with a knife on a block so that the end to end suture did not result in the best possible recovery. Statistically the method produces muscles which at maximum

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recovery have very similar degrees of partial denervation. Changes in muscle weight and muscle strength were followed for 158 days in such animals to determine the rate and extent of recovery. In similarly prepared animals a closed neurotomy was done on the gastrocnemius and the extent of muscle recovery compared with those without neurotomy. Further, a series of normally innervated muscles was subjected to closed neurotomy and the acute and chronic effect of this on fully innervated muscle so determined. The closed neurotomy was done by placing the leg of the animal upon a brass block and beating the gastrocnemius muscle through the skin with a small brass hammer driven by a 'Vibrotol'.

Muscle strength was measured with a torsion lever using optical recording. Stimuli were rectangular electrical pulses of supermaximal intensity and of a pulse duration and frequency suited to the irritability status of the nerve or muscle under test.

TABLE 1. MUSCLE CHANGES FOLLOWING NERVE SECTION AND SUTURE

NO. OF ANIMALS	TIME IN DAYS AFTER NERVE SECTION AND SUTURE	MUSCLE <sup>1</sup>					
		Weight	P <sup>2</sup>	Strength			
				Direct stimulation		Nerve stimulation	
				Per muscle	P <sup>2</sup>	Per muscle	P <sup>2</sup>
6	0	97.7		97.0		113.0	
8	14	57.7	.000	28.1	.000	0.0	.000
8	28	36.8	.000	17.2	.014	1.1	.290
7	56	50.7	.000	20.3	.027	7.1	.001
5	82	70.5	.000	44.8	.000	30.4	.000
8	91	74.9	.239	61.5	.004	52.4	.000
9	158	75.3	.922	59.6	.768	57.1	.023

<sup>1</sup> Values expressed as percentage of the contralateral unoperated control. <sup>2</sup> P for the Fisher *t* for successive differences.

Changes in muscle weight and strength in the time following nerve section and suture are shown in table 1. Weight recovery may be regarded as completed at 91 days after nerve section or about 63 days after the first functional signs of reinnervation. Permanent impairment has resulted from the nerve lesion as shown by the final weight attained.

The muscle response to direct stimulation lags behind the weight recovery. There is only a slight change in weight between the 82- and 91-day period while there is a significant increase in strength during the same time. This strength increase must be predicated on an improvement in the functional capacity of the muscle cell cytoplasm.

While the muscle strength recovery in response to direct stimulation seems to be complete at 91 days it will be noted that further increase in the muscle response to nerve stimulation occurs between the 91- and 158-day periods. From these relations in recovery it appears that the reinnervation has its first effect upon muscle weight, that functional restitution of the muscle cell cytoplasm follows this structural replacement, and that these increases in weight and strength, while dependent upon

the reestablished nerve supply, precede in time the actual achievement of motor control of the muscle by the nerve. Thus the sequence of restitution seems to detect a trophic influence of motor nerve upon muscle beyond the motor command that the nerve has over the muscle at that time.

The result of applying the neurotripsty technique to normally innervated muscle is shown in table 2. This indicates extensive acute damage to the muscle with motor nerve injury beyond the muscle damage. The gross appearance of such a muscle immediately after the beating is rather discouraging. Mascrated, pulpy and bloody,

TABLE 2. MUSCLE CHANGES FOLLOWING NEUROTIPSTY TO COMPLETELY INNERVATED MUSCLE

NO. OF ANIMALS	TIME IN DAYS AFTER NEUROTIPSTY	MUSCLE <sup>1</sup>					
		Weight	P <sup>2</sup>	Strength			
				Muscle stimulation		Nerve stimulation	
				Per muscle	P <sup>2</sup>	Per muscle	P <sup>2</sup>
6	0	101.3	.884	64.0	.027	44.0	.010
10	62	96.4	.392	99.7	.992	109.0	.300

<sup>1</sup> Values expressed as percentage of the contralateral unoperated control. <sup>2</sup> P for the Fisher for difference from unoperated contralateral control.

TABLE 3. MUSCLE CHANGES FOLLOWING NEUROTIPSTY TO PARTIALLY INNERVATED MUSCLE

NO. OF ANIMALS	TIME IN DAYS FROM		MUSCLE <sup>1</sup>					
	Nerve section to neurotripsty	Nerve section to examination	Weight	P <sup>2</sup>	Strength			
					Muscle stim.		Nerve stim.	
					Per muscle	P <sup>2</sup>	Per muscle	P <sup>2</sup>
5	25	82	103.0	.525	105.1	.506	116.2	.346
10	56	158	99.0	.936	102.2	.856	91.0	.191
8	91	158	101.7	.728	100.8	.922	93.6	.269

<sup>1</sup> Values expressed in percentage of average of muscles having same nerve cut, suture, and recovery time but no neurotripsty.

<sup>2</sup> P for Fisher t for difference between neurotripsty and non neurotripsty.

it appears that permanent damage must have been done. That these changes are completely reversible is shown by the response of such muscles after 62 days recovery. They are then the equal of their contralateral controls in all respects. From this it was concluded that such a neurotripsty could be done to produce over 50 per cent acute denervation and still allow complete recovery so that there should be no loss from the neurotripsty per se to mask any gain that might result from its application to partially denervated muscle.

The results of applying neurotripsty to partially denervated muscles are given in table 3. An early neurotripsty group was done 25 days after nerve section. At this time very little muscle reinnervation had occurred. An intermediate group received neurotripsty at 56 days, a time when very rapid reinnervation was occurring, and a late neurotripsty group was treated 91 days after nerve section when weight and

muscle strength recovery had reached a maximum. The results show that the neurotomy procedure was without effect since it neither enhanced nor retarded the extent of recovery. Thus these results are in agreement with those of Fredrick and Kossman (7). The inducement of motor nerve fibers to adopt adjacent denervated muscle fibers is an attractive objective, but it appears that the neurotomy procedure is not reliably adequate to induce motor nerve fibers to exceed their spontaneous adoption potential.

#### SUMMARY

The rate of restitution of muscle weight and strength after motor nerve section and suture has been determined. The sequence of the recovery of weight and of contractile power in response to direct stimulation and in response to nerve stimulation indicates a trophic influence of the motor nerve on the muscle beyond the ability that the nerve has for muscle excitation.

Neurotomy done at various times after nerve section and suture did not alter the final extent of muscle recovery.

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# RESPONSES ELICITED BY COMBINED STIMULATION OF PAIRS OF FIXED ELECTRODES IN THE UNANESTHETIZED MONKEY<sup>1,2</sup>

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A NUMBER of reports have been made on the effects of stimulation of the cerebral cortex in the unanesthetized animal (1, 2) but we have found none concerning the effects of concurrent or immediately consecutive stimulation of two or more cortical points in such preparations despite the fact that such studies have been fruitful in the anesthetized animal. The effects of concurrent or successive stimulation of two points are of special interest in view of the problems raised by the discovery of the so-called suppressor bands (3, 4). The following report is concerned with the influence of stimulation of the anterior suppressor band upon the excitability of other cortical regions in the unanesthetized monkey.

## METHODS AND RESULTS

Electrodes were implanted aseptically in 10 immature macaque monkeys under nembutal anesthesia (2). Figure 1 shows the right hemispheres of these monkeys, upon which the sites of the stimulating electrodes are marked. In the first diagram (*monkey 18*), the positions of all the electrodes implanted in that hemisphere are given, while in the remaining monkeys only the electrodes used in this study are shown. Even numbers indicate that the electrode was located on the right hemisphere, the odd numbers on the left. On the day following the operation, when the animals were awake and alert, they were placed in an examination chair and stimulated with 60 cycle sine wave current derived from the lighting circuit. The specific pairs of electrodes to be used as well as the required current strengths were decided after study of the responses elicitable from each electrode separately. This portion of the work has already been reported (1).

It is difficult to place an electrode on the anterior suppressor band accurately

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from surface markings of the skull. Since, however, Bailey, *et al.* (5) found that the anterior suppressor band was closely related to area 8, the region giving head and eye movements, we chose for further study those electrodes eliciting such movements in a given animal from the several implanted in that general region. With such an electrode as one of a pair in each experiment, another electrode on the same side usually, which gave either arm or leg movements was used.

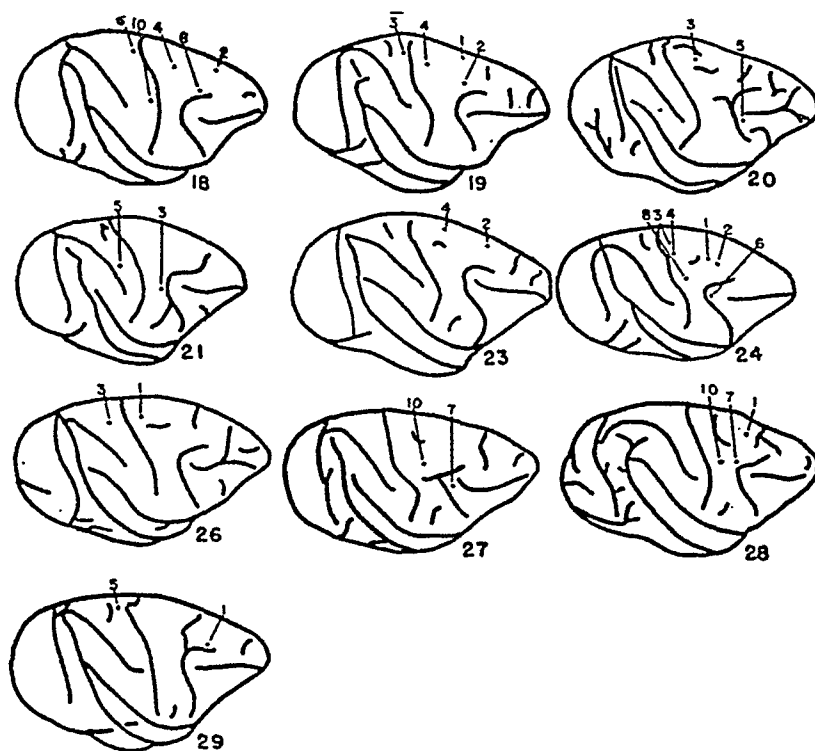


FIG. 1. DIAGRAMS SHOWING SITES OF IMPLANTED ELECTRODES

A condensed protocol giving pertinent data from one of these experiments (*monkey 23*) follows:

ELECTRODE	VOLTAGE	DURATION	RESPONSE
2	0.8	4 sec.	Head turned to left but no eye movements were observed.
4	0.8	4 sec.	With about $\frac{1}{2}$ second latency the leg was flexed.
Both	0.8	4 sec.	With the stimulus the left hind leg was flexed and head began to turn to left. Leg did not relax for 20 seconds.

The results are summarized in table 1. Following the number of each animal are 4 columns indicating the location of the primary movement (as in eye, head, arm or leg) elicited from each of the particular electrodes selected for combined stimulation. In many cases movements in more than one of these locations occurred with the particular strength of current used. Thus in *monkey 26* stimulation by electrode 1 produced movements in head, arm and leg. The order of stimulation is indicated by the symbols S—simultaneous, C—consecutive (routinely in consecutive stimulation the first electrode activated was the one from which head or head and eye movements

could be elicited), CB—where both orders were used and B—both consecutive and concurrent stimulation. Any change in the response is indicated in the following column. It is noteworthy that all changes in duration, amplitude or in extent of movement were toward an augmented response and in no case did the reverse occur. Clonus occurred in many animals at the current strengths selected. In several of these clonus occurred with the combined stimulation but was not present in the

TABLE 1. SUMMARY OF RESULTS

MONKEY NO.	PRIMARY MOVEMENT PRELIMINARY STIMULATION				ORDER OF STIMULATION	CHANGES IN RESPONSE	CLONUS	
	Head	Eye	Arm	Leg			Preliminary stimulation	Combined stimulation
18			4	6	S	In	Yes	Yes
19			1	3	S	In	No? <sup>1</sup>	Yes
	x <sup>2</sup>	x <sup>2</sup>	2, 4	2, 4	S	In	Yes	Yes
20	5	5		3	S	None	Yes	Yes
21	3		3	5	S	In	No	Yes
23	2	2		4	S	In	No	Yes
24	2		2	4	B	In? <sup>3</sup>	Yes	Yes
				1, 3 <sup>4</sup>	S	In	No	No
	6		6, 8		C	In	No	No
26			1, 3		CB	In	No	Yes
27	7	7	10		C	In	Yes	Yes
28	1	1	10		C	None	Yes	Yes
	7		10		CB	None	Yes	Yes
29	1			5	B	In	No	? <sup>5</sup>

<sup>1</sup> At intensity of stimulation used in combined stimulation clonus appeared inconstantly during preliminary stimulation.

<sup>2</sup> There were no head and eye movements during preliminary stimulation but they did appear as part of the response to combined stimulation. This is indicated by the x in those columns.

<sup>3</sup> This increase in response may be doubtful but if any change can be considered it was in this direction.

<sup>4</sup> The voltage used for electrode 1 was below threshold, nevertheless knee flexion (the primary movement from electrode 1) was present. The response from electrode 3 was primarily inversion and dorsiflexion of the foot.

<sup>5</sup> There was a clonic jerk following the response to combined stimulation and 3 such jerks when there was a 4-second delay between the 2 stimuli. However, with consecutive stimuli (at a slightly lower voltage) there was no evidence of clonus.

earlier single stimulation at the same intensity. The opposite effect was not seen. It should be emphasized that there was never any indication of inhibition either of movement or of after discharge. In summary, the responses to combined stimulation of two points were augmented as compared with the responses obtained from separate stimulation in 11 of the 14 attempts.

Aside from the possibility that stimulation of a suppressor band might have produced unconsciousness in an unanesthetized patient (6, 7), there are two responses which study of the literature would lead one to anticipate eliciting under the conditions of our experiments. These are: 1) a holding in abeyance of a cortical after discharge and 2) suppression of a motor response. The latency of the first of these is

very short even in the anesthetized animal where electrical stimulation of a suppressor band is said to inhibit immediately the clonic after discharge so readily produced by supraliminal stimulation of the motor cortex. One could have reasonably expected that mild clonus at least would have been inhibited rather than facilitated in the nonanesthetized animal by suppressor band stimulation. Since McCulloch (3) has shown that the duration and latency of suppression of a motor response are functions of the depth of anesthesia, it could be expected that such responses would have a very short latency in the unanesthetized animal and that suppression of a motor response should therefore have been apparent under the conditions of our experiments.

Two explanations may be advanced for the results obtained. It is possible that suppression is an artifact that can be elicited only under definitely abnormal conditions. Some support for this view may be gleaned from the literature. For example, how can one interpret the statement of Gellhorn (8) that "suppression is most easily elicited 18 or more hours after the operation" (his animals were under continuous Dial anesthesia) as other than that abnormal conditions must prevail in order to demonstrate this phenomenon. Furthermore, Clark, *et al.* (9) report that they were able to elicit suppression in dogs anesthetized with Dial but were unable to do so in the same animals when they were awake and alert. Since these workers used implanted electrodes, constant points were stimulated. When the present report of facilitation rather than suppression in the unanesthetized monkey is added to this, a rather strong case can be made. On the other hand, we are inclined to the view that both facilitation and suppression (4) can be elicited from the same cortical area, and that separation of these responses is possible under proper conditions. Under Dial anesthesia facilitative responses may be selectively eliminated as was shown in the dog by Clark, *et al.* (9). With other types of anesthesia suppression may be demonstrated with difficulty while in the unanesthetized animal facilitative responses may completely mask suppression. Such a statement, however, gives no clue concerning the rôle, if any, which suppression plays in the intact animals.

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# EFFECT OF CARBON DIOXIDE ON BRAIN GLUCOSE, LACTATE, PYRUVATE AND PHOSPHATES<sup>1,2</sup>

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CENTRAL nervous activity is considerably affected by procedures that cause changes within the carbon dioxide system of blood and tissue. For example, respiration of suitable concentrations of carbon dioxide induces hyperpnea, convulsions and anesthesia. Hyperventilation tends to induce relatively high-voltage, low-frequency cortical potentials and, in petit mal, typical seizure discharges (1). Conversely, petit mal seizures can be interrupted by increasing the carbon dioxide concentration of the inspired air (2). The total carbon dioxide content of arterial and jugular blood fluctuates abnormally prior to the onset of seizures (3). Repeated breathing of high concentrations of carbon dioxide appears to relieve certain neuroses (4). Moreover, breathing gas mixtures containing large (15-30%) amounts of carbon dioxide raises the convulsive thresholds to certain drugs and to electric shock, whereas it lowers the convulsive threshold to other drugs (5, 6).

At present there is insufficient information to warrant extensive consideration of the basic cellular mechanisms whereby the carbon dioxide system might affect central nervous activity. It appears that, due to relatively rapid rates of diffusion, the carbon dioxide and carbonic acid concentrations of the blood are the primary determinants of the hydrogen ion concentration of muscle (7). Generalization from this supports the impression that central control of respiration is more responsive to blood levels of carbon dioxide and carbonic acid than to the level of hydrogen ions (8). The central presence and localization of carbonic anhydrase (9) and the pathological variations in its concentration (10) suggest a special rôle for the carbon dioxide system in central nervous metabolism. The increase in cerebral blood flow (11) and consequent increase in oxygen tension of cortex (12) brought about by breathing increased concentrations of carbon dioxide and the decrease in flow (11) and oxygen tension (12) induced by hyperventilation are variables pertinent to considerations of central effects of the carbon dioxide system

In the present work, in order to gain information that might further rational consideration of the rôle of carbon dioxide in central nervous metabolism, the cerebral hemispheres of cats were assayed for glucose, lactate, phosphates and pyruvate following changes which were effected in several ways within the carbon dioxide system.

## EXPERIMENTAL

*Procedures.* Cats were employed as the experimental animals. They were paralyzed with dihydro- $\beta$ -erythroidine, maintained with artificial respiration, and the

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skulls exposed for eventual freezing of the brain. One hour after onset of paralysis the animals were subjected to particular experimental procedures at the end of which the brains were frozen by pouring liquid air on the exposed skulls. Arterial blood was taken in heparin at appropriate times. The electrical activity of heart and brain was recorded during the experiments with a six-channel Grass Electroencephalograph. The EEG leads were taken from screw type electrodes placed bilaterally over the center of the cerebral cortex. Blood gases were measured manometrically with the usual Van Slyke apparatus. The hydrogen ion concentration of blood was measured with the Beckman  $pH$  meter, model G, using Model 290J electrodes to prevent escape of carbon dioxide. The preparation of the animals and other methods of assay have been reported in detail (13, 14).

*Lactate and Pyruvate.* The effects of various experimental conditions upon the concentrations of brain lactate and pyruvate are indicated in table 1. The highest level of lactate was found in the animals having the lowest level of blood carbon dioxide (*exper. 1*) and the lowest level in those breathing carbon dioxide plus 20 or more per cent oxygen (*exper. 8-14*). Intermediate levels were found in animals not given carbon dioxide, but having blood carbon dioxide contents higher than in *experiment 1* (*exper. 2-6*). The pyruvate levels in the animals given bicarbonate, carbon dioxide or both were lower than in those given no special treatment (compare *exper. 1-4* with *5-15*).

The effect of breathing carbon dioxide on the concentrations of brain lactate and pyruvate was evident with 5 per cent (*exper. 8*), although not consistently (*exper. 7*), and was somewhat greater with higher concentrations. Administration during two minutes was sufficient to induce the lower levels of lactate and pyruvate (*exper. 10*).

The concentration of brain lactate bears some inverse relation to blood carbon dioxide levels (*exper. 1-5*); however, comparison of the lactate concentrations obtained when bicarbonate was administered (*exper. 5, 6*) with the concentrations found when carbon dioxide was given (*exper. 8-14*) indicates an exception to this apparent relation. The brain lactate level obtained when bicarbonate was administered alone (*exper. 5*) was higher than when carbon dioxide was also given (*exper. 6*). This, and the finding that administration of carbon dioxide alone resulted in the lowest lactate levels, suggests an inverse relation between the hydrogen ion concentration of blood and the concentration of brain lactate. However, the hydrogen ion concentration of blood cannot be the only determinant of brain lactate since the relatively high concentration of hydrogen ions in blood produced by injection of hydrochloric acid (*exper. 18*) was not accompanied by a particularly low level of brain lactate.

The concentration of blood oxygen may be concerned in the determination of the lactate level, since reduction of the oxygen content of the gas breathed to 10 per cent produced an elevation of lactate concentration (compare *exper. 9* and *15*). Breathing 10 per cent oxygen alone presumably would not affect the concentration of brain lactate (14).

Carbon dioxide in relatively high concentration did not prevent the increase in brain lactate expected to accompany a suitable degree of hypoxia (*exper. 16, 17*).

TABLE 1. EFFECT OF CO<sub>2</sub> ON BRAIN LACTATE AND PYRUVATE<sup>1</sup>

EXPER. NO.	CONDITIONS	BRAIN		FINAL BLOOD		
		Lactate	Pyruvate	CO <sub>2</sub>	pH	O <sub>2</sub> Sat.
1	Room air	2.8 ± 0.2 (2)	0.22 ± 0.09 (2)	30 ± 3 (2)	7.65 ± 0.20 (2)	0.97 ± 0.02 (2)
2	Room air	2.2 ± 0.5 (13)	0.22 ± 0.11 (12)	32 ± 3 (3)		0.98 ± 0.03 (3)
3	Room air	1.4 ± 0.3 (6)	0.20 ± 0.08 (4)	47 ± 4 (4)		0.86 ± 0.07 (4)
4	100% O <sub>2</sub> ; 10 min.	1.7 ± 0.3 (4)	0.21 ± 0.06 (4)	36 ± 6 (4)	7.33 ± 0.12 (4)	0.99 ± 0.03 (4)
5	I.v. bicarb.; room air	1.4 ± 0.2 (3)	0.12 ± 0.05 (2)	148 ± 50 (3)	7.84 ± 0.17 (3)	0.96 ± 0.03 (3)
6	I.v. bicarb.; 15% CO <sub>2</sub> , 85% O <sub>2</sub> ; 10 min.	1.2 ± 0.1 (2)	0.11 ± 0.01 (2)	158 ± 46 (2)	7.42 ± 0.02 (2)	1.08 ± 0.03 (2)
7	5% CO <sub>2</sub> , 95% O <sub>2</sub> ; 5 min.	1.8	0.19	44	7.07	0.98
8	Same as 7	0.9	0.13	46	7.29	1.00
9	15% CO <sub>2</sub> , 85% O <sub>2</sub> ; 10 min.	0.6	0.12	70		1.00
10	20% CO <sub>2</sub> , 80% O <sub>2</sub> ; 2 min.	0.8	0.07	61	6.99	1.00
11	20% CO <sub>2</sub> , 80% O <sub>2</sub> ; 5 min.	0.7 ± 0.1 (3)	0.07 ± 0.01 (3)	58 ± 5 (3)	6.94 ± 0.03 (3)	1.00 (3)
12	20% CO <sub>2</sub> , 80% O <sub>2</sub> ; 10 min.	0.4	0.06	56	6.92	1.00
13	20% CO <sub>2</sub> , 80% O <sub>2</sub> ; 20 min.	0.8	0.06	62	6.95	0.95
14	30% CO <sub>2</sub> , 70% O <sub>2</sub> ; 10 min.	0.6	0.09	60		1.00
15	15% CO <sub>2</sub> , 10% O <sub>2</sub> , 75% N <sub>2</sub> ; 10 min.	1.4 ± 0.05 (2)	0.11 ± 0.01 (2)	57 ± 7 (2)		0.37 ± 0.08 (2)
16	15% CO <sub>2</sub> , 6% O <sub>2</sub> , 79% N <sub>2</sub> ; 10 min.	9.6	0.26	57		0.25
17	100% CO <sub>2</sub>	8.32		106	6.50	0.04
18	I.v. HCl; 7 min.	1.5		32	7.10	0.69
19 <sup>2</sup>	15% CO <sub>2</sub> , 85% O <sub>2</sub> ; 10 min.	1.7 ± 0.1 (3)	0.14 ± 0.03 (3)			
20	15% CO <sub>2</sub> , 85% O <sub>2</sub> ; cyanide i.v.	5.6		38		1.02
21	Convulsions; room air	6.2	0.22 ± 0.11			
22	Convulsions; room air	5.0 ± 0.7 (2)	0.17 ± 0.07 (2)			
23	Convulsions 15% CO <sub>2</sub> , 85% O <sub>2</sub>	2.2 ± 0.6 (5)	0.18 ± 0.09 (3)	60 ± 4 (5)	7.01 ± 0.06 (3)	1.02 ± 0.050 (5)
24	Convulsions 15% CO <sub>2</sub> , 10% O <sub>2</sub> , 75% N <sub>2</sub>	2.5 ± 0.1 (2)	0.10 ± 0.02 (2)			

<sup>1</sup> Where appropriate, 3 figures representing the mean, standard deviation and no. of animals (in parentheses) are given for each assay in a particular experiment. Concentrations of lactate and pyruvate are expressed in mm/kg. of tissue, and CO<sub>2</sub> content in volumes %. O<sub>2</sub> saturation signifies the ratio of the O<sub>2</sub> content of the blood as drawn to that after equilibration with room air. The data for lactate and pyruvate in *exper. 2* and *21* have been reported previously (13). *Exper. 1-3* differ in rates of ventilation used. In *exper. 5* and *6*, 60 ml. of 0.5 M sodium bicarbonate was injected intravenously during the period of time indicated. In *exper. 6* administration of CO<sub>2</sub> was begun immediately after initiation of bicarbonate injection. In *exper. 18*, 80 ml. of 0.05 M HCl was injected intravenously during the period indicated. In *exper. 20*, 0.8 mg. sodium cyanide/kg. body weight was injected immediately after the animal began breathing CO<sub>2</sub>. Convulsions were produced by intravenous injection of about 15 mg. metrazol/kg. body weight. Brains frozen at end of 180 seconds of convulsive activity essentially like that recorded in (13).

<sup>2</sup> Carotids ligated immediately after the animals began breathing CO<sub>2</sub> mixture.

and 20). The concentration of lactate found when the carotids were ligated during administration of carbon dioxide (*exper. 19*) was higher than when carbon dioxide was given alone. Therefore, it appears that cerebral circulation is involved in the effect of carbon dioxide on the concentration of lactate. It should be pointed out here that in the cat cerebral function can be maintained by the vertebrals alone.

The foregoing remarks about lactate apply to brain pyruvate, although in lesser degree.

Summarizing, it appears that the concentrations of brain lactate and pyruvate bear an inverse relation to the levels of blood carbon dioxide, hydrogen ions, oxygen and cerebral blood flow.

TABLE 2. EFFECT OF CO<sub>2</sub> ON BRAIN PHOSPHATES AND GLUCOSE<sup>1</sup>

EXPER. NO.	CONDITIONS	BRAIN					BRAIN/PLASMA GLUCOSE
		IP	PC	ATP	ADP	Glucose	
1	Room air	4.7± 1.8 (14)	2.2± 0.5 (14)	1.3± 0.5 (10)	1.1± 0.7 (10)	4.2± 1.1 (11)	0.36± 0.08 (11)
2	Room air	4.2± 1.3 (7)	2.6± 0.6 (9)	1.5± 0.6 (7)	0.9± 0.5 (7)	3.9± 1.2 (10)	0.38± 0.02 (10)
3	Bicarbonate	3.9± 0.9 (5)	3.0± 0.5 (5)	1.7± 0.2 (5)	0.7± 0.1 (5)	3.8± 0.6 (5)	0.43± 0.07 (5)
4	CO <sub>2</sub>	4.3± 0.6 (5)	2.2± 0.1 (7)	1.4± 0.1 (7)	0.9± 0.1 (7)	4.9± 1.9 (10)	0.33± 0.05 (10)
5	Convulsions; room air	5.8	1.7	0.6	2.1	1.9	0.21
6	Convulsions; room air	6.0± 0.7 (2)	0.8± 0.3 (2)	0.9± 0.2 (2)	1.9± 0.6 (2)		
7	Convulsions; 15% CO <sub>2</sub> , 85% O <sub>2</sub>	3.6± 0.5 (4)	2.1± 0.2 (4)	1.4± 0.2 (4)	0.7± 0.2 (4)	5.5± 2.0 (5)	0.26± 0.07 (5)

<sup>1</sup> Concentrations are expressed in mm/kg. Portions of the data for *exper. 1* and *5* have been reported previously (13). The animals used in *exper. 2* were those of *exper. 1, 3, and 4*, table 1; in *exper. 3* were those of *exper. 5 and 6*, table 1; in *exper. 4* were those of *exper. 8-14*, table 1. The animals used in *exper. 7* were those of *exper. 24*, table 1.

*Acid-soluble Phosphates and Glucose.* The concentration of brain phosphates, glucose and ratio of brain to plasma glucose under various experimental conditions are given in table 2. The results for *experiments 2-4* were derived from data obtained in relatively similar individual experiments that differed in certain details. Comparison between the individual experiments was made but revealed no notable differences.

The data indicate that neither intravenous injection of considerable amounts of bicarbonate nor respiration of relatively high concentrations of carbon dioxide had any appreciable effect on the concentrations in brain of inorganic phosphate, phosphocreatine, adenosine phosphates or glucose.

*Convulsions.* Data indicating the effect of breathing carbon dioxide on the changes in brain accompanying seizures are given in tables 1 and 2. As shown by comparison of *experiments 2* with *19 and 20*, table 1 and other earlier work (13, 14, 16), convulsions are accompanied by an increase in brain lactate and by decreases

in the concentration of brain high-energy phosphates and glucose. (Compare *exper. 1* with 5 and 6, table 2.)

Comparison of *experiments 9* and 23, table 1, shows that seizures occurring during administration of carbon dioxide were also accompanied by an increase in brain lactate. The percentage increase was about the same as found in air; however, the actual increase was considerably less. Further, the increase in lactate accompanying convulsions in animals breathing carbon dioxide and 10 per cent oxygen was less (compare *exper. 15* and 22) than in animals breathing room air.

Comparison of *experiments 4* and 7, table 2, indicates that seizures occurring during administration of carbon dioxide, in contrast to the results in room air (compare *exper. 1* and 2 with 5 and 6), were not accompanied by appreciable change in the concentrations of high-energy phosphates.

Although the concentration of brain glucose in the convulsed animals breathing carbon dioxide was higher than in the controls (compare *exper. 4* with 7), the ratio of brain to plasma glucose was lower. Since, in the absence of rapid change in rate of metabolism or rate of supply of glucose, the ratio of brain to plasma glucose is relatively constant (*exper. 1-4*) (17), this decrease in ratio may represent a decrease in concentration of brain glucose effected by the seizures.

Summarizing, breathing carbon dioxide practically prevents the decrease in high-energy phosphates accompanying seizures, limits the increase in lactate, and has little effect on the decrease in brain to plasma glucose ratios.

#### DISCUSSION

It is known that breathing carbon dioxide increases the rate of cerebral blood flow and that hyperventilation decreases the rate of flow (11) and the change in cerebral oxygen tension is in the expected direction (12). The rates of blood flow during administration of carbon dioxide, in the control state, and during hyperventilation are in arbitrary units about 4, 2, and 1 respectively (11). The concentrations of brain lactate in what roughly corresponded to those several states were about 0.7, 1.4, and 2.8 mM per 1000 grams respectively (compare *exper. 8-14*, 3 and 1, table 1). This nice inverse correspondence between probable rates of blood flow and concentrations of brain lactate, admittedly fortuitous, and the observation that ligation of the carotids limits the fall in lactate level accompanying administration of carbon dioxide suggest a major rôle for blood flow in determining the concentrations of lactate and, to a lesser extent, pyruvate in brain. Thus, it may be proposed that all of the present procedures that resulted in relatively low levels of brain lactate and pyruvate mediated their effect by producing a high rate of blood flow and, conversely, those resulting in high levels (except seizures) did so by effecting a relatively low rate of flow.

Blood flow might act by changing the effective concentrations of these substances in blood, e.g., an increase in rate of blood flow would be comparable to decreases in concentration of the substances in blood. From this, some relation between the concentrations in blood and brain would be expected. However, no consistent relation between blood and brain concentrations was found, and the available evidence indicates that markedly high levels of blood lactate have little, if any, influence on

the concentration in brain (18). The same low levels of brain lactate and pyruvate, induced by administration of carbon dioxide, were found in the presence of two-fold differences in blood levels. Animals breathing air and having high levels of brain lactate and pyruvate had blood levels lower than in animals breathing carbon dioxide and having, consequently, low concentrations of these substances in brain. Thus, the essentially mechanical aspects of blood flow hardly suffice as explanation of its relation to brain lactate and pyruvate.

The oxygen tension of cerebral cortex varies directly with blood flow (11). The finding that decrease in the concentration of oxygen in the carbon dioxide mixture breathed to 10 per cent limited the fall in lactate and pyruvate levels induced by the carbon dioxide suggests that the oxygen tension of brain may inversely affect the brain levels of the substances in question. In the case of lactate such an effect might proceed by varying the concentration of reduced diphosphopyridine nucleotide available for reduction of pyruvate, e.g., high oxygen tension would decrease the concentration of reduced nucleotide and consequently the rate of formation of lactate from pyruvate.

In addition, the level of pyruvate available for reduction might be reduced by increased efficiency of the Krebs cycle at increased oxygen tensions. There is a suggestion of this in the data since pyruvate drops with carbon dioxide and oxygen mixtures. It does not drop with 100 per cent oxygen but there is only a small increase in oxygen tension of the cortex under these conditions compared to carbon dioxide plus oxygen (6).

There is another related rationale for the observed changes in lactate under carbon dioxide inhalation. Brain is reported to have an unusually high rate of aerobic glycolysis (19). It has been shown in rat liver that one in eight carbons of newly formed glycogen comes from bicarbonate (20) and it was proposed that the glycogen was formed from carbon dioxide and phosphopyruvic acid. Moreover, it is known that carbon dioxide can be assimilated (21, 22). Presumably these reactions can also occur in brain since it has been demonstrated that carbon dioxide is incorporated into citrate in brain *in vivo* (23). All of these reactions would have the effect of limiting the amount of pyruvate available to accept hydrogen from the glycolytic mechanisms and might result in lowered lactates.

Craig (24) has reported that increase in hydrogen ion concentration and total carbon dioxide (from 1 per cent to 5 per cent) results in an increase in the rate of aerobic glycolysis in brain slices *in vitro*. Levels of carbon dioxide above 5 per cent had no further effect. The present work is not in obvious agreement with this report, but it should be noted that the level of carbon dioxide in the residual alveolar air is approximately 5 per cent (25). The exact carbon dioxide tension of the cortex in these artificially ventilated animals is not known although the value of 30 volumes per cent in the arterial blood is suggestive of values considerably above 1 per cent. It would appear that in the *in vitro* studies (24) changes were noted at values below normal carbon dioxide levels but not above.

There is little doubt that carbon dioxide has profound effects upon the levels of lactate, pyruvate and phosphates in the brain. The mechanism by which these effects are mediated remains obscure. The most probable explanation is that the

resulting increase in blood flow and oxygen tension enabled the brain to more nearly balance energy demands with oxidative processes and as a consequence less lactate was formed and high energy phosphate reservoirs were not depleted. The effects are especially marked during convulsions where energy demands are high. The other possibilities discussed above may contribute to the overall effect, but it is the authors' feeling that theirs is a minor rôle.

#### SUMMARY

The brains of normal and convulsing cats subjected to conditions designed to alter the carbon dioxide—bicarbonate—carbonic acid system were analyzed after freezing with liquid air. In normal animals, breathing 10 to 30 per cent carbon dioxide mixtures resulted in a lowering of lactate and pyruvate levels and had little effect on glucose or phosphate concentrations. In convulsed animals these conditions markedly limited the expected rise in lactate, pyruvate and inorganic phosphate and prevented the fall in high-energy phosphates. There was a rise in brain glucose but little effect was found on brain/plasma glucose ratios. The results are discussed with relation to possible mechanisms and the effects of carbon dioxide on the central nervous system.

The authors wish to acknowledge with thanks the painstaking technical assistance of Miss Ruth Hurwitz and Mrs. Stiscie Cutler.

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## CENTRAL CONNECTIONS FOR AFFERENT FIBERS FROM THE KNEE JOINT OF THE CAT<sup>1</sup>

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**N**ERVES supplying the knee joint of the cat contain myelinated fibers, some of which are as large as 16 or 17 microns in diameter (1). The fiber spectrum resembles that found in cutaneous nerves. Most, if not all, of the myelinated fibers are afferent in nature and enter the spinal cord over 3 and sometimes 4 dorsal roots (2). Their subsequent connections have not until now been studied experimentally and this report presents data concerning pathways in the spinal cord for impulses initiated in articular fibers.

### METHODS

Cats were used in all the experiments. Two were decerebrated under ether anesthesia, while 15 were anesthetized with sodium pentobarbital given intravenously or intraperitoneally. The articular branches of the tibial and saphenous nerves, designated respectively as posterior and medial branches, were used. Two types of experimental procedures were carried out: 1) Articular nerves were stimulated and potential changes recorded from the dorsum of the spinal cord. In several experiments, attempts were also made to record from lateral funiculi. 2) Dorsal funiculi were stimulated and antidromically conducted impulses recorded from articular nerves.

The spinal cord and hind-limb structures were covered with warm mineral oil. Silver wire was used for stimulating and recording electrodes. Potentials were amplified, led to a cathode ray oscillograph and photographic records were usually taken.

### RESULTS

*Stimulation of Articular Nerves.* When the posterior nerve was stimulated with single shocks, potential changes of relatively long duration were recorded from the

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dorsum of the spinal cord over a distance of 30 to 40 millimeters (fig. 1). Most of the articular fibers enter the cord over the 6th and 7th lumbar dorsal roots, to a lesser extent over the 5th lumbar root, and occasionally over the 1st sacral root as well. The slow potentials were usually maximal at the 6th and 7th lumbar levels and exhibited well-defined negative and positive phases. Cranially and caudally the positive phase disappeared rapidly, and the negative phase, while detected over a greater extent, sometimes 6 or 7 segments, changed in amplitude and slope characteristics (fig. 1). These slow potentials, while little affected even by deep anesthesia, were quite sensitive to asphyxia.

Spike potentials preceding the negative phase were not seen, except when using an amplifier with high frequency response, and then not uniformly. When present, they were small and could be detected only at levels of entry. The fastest of them conducted at rates of 90 to 100 meters per second.

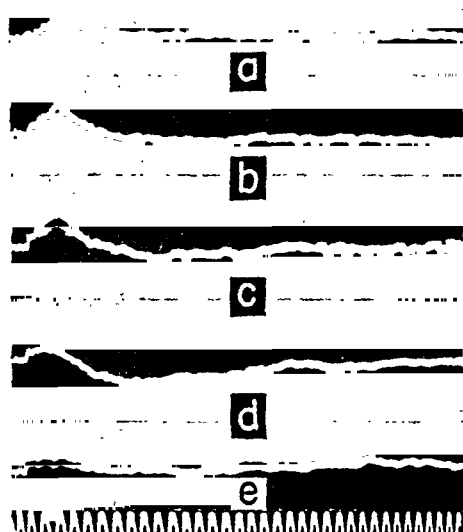


Fig. 1. SLOW POTENTIALS recorded from surface of ipsilateral dorsal funiculus after single volleys over posterior nerve. Electrodes were bright silver wire, one placed on the dorsum of the cord, the other thrust into adjacent musculature. *a*, Potential recorded at the 3rd lumbar segment; *b*, 4th lumbar segment; *c*, 5th lumbar segment; *d*, 6th lumbar segment; *e*, junction of 7th lumbar and 1st sacral segments. Distance between *a* and *e*, 40 millimeters. Time in 4 msec. intervals.

Most of the experiments utilized the posterior nerve. Comparable studies of the medial nerve in two experiments gave similar results, although the potentials were somewhat greater in magnitude.

All attempts to record spikes ascending in dorsal funiculi at more cranial levels were unsuccessful. The articular nerves are small so that relatively few funicular fibers are activated by stimulation of an articular nerve. Potentials are probably shunted, and increasing amplification merely tends to emphasize spontaneous activity. In a few experiments, attempts were made to record from the surface of the dorsal spinocerebellar tracts at upper lumbar and midthoracic levels, but these attempts were uniformly unsuccessful. Systematic exploration of these tracts with microelectrodes was not carried out, however, nor were recordings attempted in decerebrate preparations.

*Stimulation of Dorsal Funiculi.* With stimulation of dorsal funiculi, antidromically conducted impulses were readily recorded from articular nerves. Because of long conduction distances, there was considerable temporal dispersion (fig. 2). Only the initial deflections were used in the determination of conduction rates,

mainly because of possible complication by dorsal root reflexes (3). Section of ipsilateral dorsal funiculi below the level of stimulation abolished the responses, which could then be obtained by stimulation below the incision.

The maximum conduction rates observed at the levels of entry of articular fibers were 90 to 100 meters per second, but these rates decreased rapidly within the next few cranial segments so that at thoracic level the fastest rates observed were 40 to 60 meters per second. Still further decrease subsequently occurs, since at the first cervical segment the maximum rates were but 20 to 30 meters per second. The potentials recorded after stimulation at rostral levels were also smaller and much simpler in form (fig. 2).

Again, most of the experiments utilized the posterior nerve. In the two experiments involving the medial nerve, similar results were obtained and the maximum conduction rates observed were approximately the same.

#### DISCUSSION

Since Gasser and Graham's report (4), negative intermediary potentials of the type recorded in the present study have been attributed to internuncial activity. It may be assumed, therefore, that when articular fibers enter the spinal cord they give rise to a typical collateral formation by which synaptic connections are made, not only in immediately adjacent gray matter but also in that of more cranial and caudal segments. The longitudinal extent of gray matter activated by articular nerve fibers cannot be determined exactly, however, since internuncial activity at a particular level can probably be detected one or two segments away from that level. The segments to which articular fibers give collaterals may therefore be fewer in number than the 6 or 7 from which cord potentials could be recorded. Evidence of discharge over ventral roots is needed to localize the levels of synaptic connections which can be activated by single volleys.

Results obtained by stimulation of dorsal funiculi show that some articular fibers ascend in ipsilateral dorsal funiculi as far as the medulla oblongata. The rates of conduction over such fibers indicate that this ascending path is formed by processes of the larger myelinated fibers in articular nerves. Previous studies have shown that such fibers arise mainly from Ruffini-type endings in certain regions of the joint

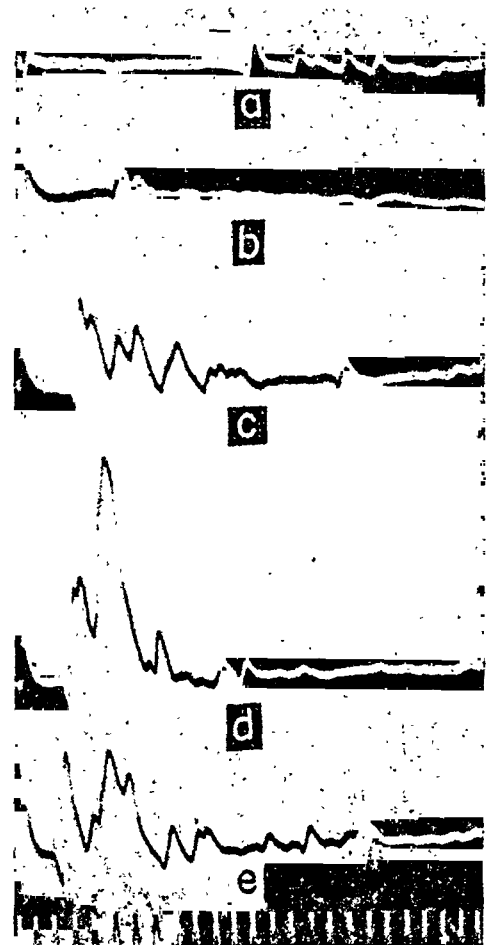


Fig. 2. ANTIDROMICALLY CONDUCTED IMPULSES recorded from the posterior nerve after stimulation of ipsilateral dorsal funiculus with bipolar silver electrodes. *a*, Stimulation of 1st cervical segment, conduction distance 489 mm.; *b*, thoracic cord, 319 mm.; *c*, 3rd lumbar segment 241 mm.; *d*, caudal part of 4th lumbar segment, 221 mm.; *e*, junction of 7th lumbar and 1st sacral segments, 181 mm. Time in msec.

and that most of them are 7 to 10 microns in diameter (1). A few are large enough to be classified as *Group I* fibers, but whether they are comparable in their anatomical connections to *Group I* fibers in muscle nerves could not be determined in this study.

The rapid decrease in conduction rates immediately rostral to levels of entry undoubtedly results from a decrease in diameter of the ascending fibers as collaterals are given off. Since a further decrease in conduction rate occurs in the cervical region, it is possible that collaterals are also given off here. The ascending fibers presumably relay in the medulla oblongata, but no studies of this feature have as yet been made. If articular nerves and their connections contribute in any way to position sense, impulses may well reach the cerebral cortex by this pathway.

Little is known concerning other ascending pathways. No positive evidence of conduction over the spinocerebellar tracts was obtained. Indirect evidence from previous experiments indicates that some articular fibers are concerned with pain mechanisms. Changes in respiration, pulse and blood pressure following stimulation of articular nerves resulted when such stimulation was strong enough to activate the smaller, more slowly conducting fibers (5). While no direct evidence is available, rostral conduction, after transmission through gray matter, no doubt would be mainly over lateral spinothalamic tracts.

In most of the experiments reported here the type and depth of anesthesia was such that reflex muscular responses were minimal or absent. Reflex activation of skeletal muscle following stimulation of small articular nerves may be readily studied only in decerebrate or decapitate preparation, and results of such studies will be reported in a later publication.

#### SUMMARY

Connections in the spinal cord of afferent fibers from the knee joint of the cat were studied as follows: 1) Articular nerves were stimulated by single shocks and the resulting activity recorded from the spinal cord. 2) Dorsal funiculi were stimulated at various levels and antidromically conducted impulses recorded from articular nerves.

The results indicate that most, if not all, articular fibers synapse with internuncial neurons shortly after entering the spinal cord. The larger myelinated fibers, those arising from Ruffini-type endings, also continue rostrally in ipsilateral dorsal funiculi to the medulla oblongata. Maximum conduction rates at levels of entry were 90 to 100 meters per second, but only 20 to 30 meters per second at cervical levels. The decrease probably results from a decrease in diameter of the parent fibers as collaterals are given off. No evidence of conduction over the spinocerebellar tracts was obtained.

It is a pleasure to acknowledge the technical help of Mr. Darwood Hansen, who is responsible for the design, construction and operation of much of the experimental equipment.

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# EFFECTS OF OXYGEN DEPRIVATION UPON THE COCHLEAR POTENTIALS<sup>1</sup>

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THE basic problem of the effects of oxygen want upon bodily functions has received an ever-increasing amount of attention in recent years, to a large extent as a result of the continued development of aerial flight at high altitudes (1). A part of this problem, which is our concern here, is the behavior of the ear and, more particularly, the action of the cochlea as revealed in its electrical potentials.

Only a few studies have dealt with hearing during oxygen deprivation and their results have been conflicting. Aggazzotti in 1904 (1a) reported a dulling of threshold acuity to sounds as a result of a reduction of atmospheric pressure. Lewis (2) in 1918 and Bagby (3) in 1921, however, observed no changes when the oxygen intake was reduced to the point of psychological failure and collapse.

Gelhorn and Spiesman (4) in 1936 reported significant reductions of sensitivity from moderately severe deprivations. They determined the thresholds of hearing, mostly for a tone of 1024 cycles, while a subject was allowed to breathe air that had been diluted with nitrogen, and in different tests varied the dilutions and periods of exposure. In a typical test they found that a mixture containing 10 per cent of oxygen (which is the equivalent, in terms of the partial pressure of oxygen exerted upon the alveoli of the lungs, of an altitude of about 19,000 ft.) when breathed for 10 to 30 minutes reduced the acuity by a significant amount.<sup>2</sup>

McFarland (5) in 1937 used a 2-A audiometer to measure the acuity of 6 subjects at sea level and after an ascent of the Andes mountains to an elevation of 17,500 ft. (equivalent to an atmosphere at sea-level pressure containing 10.7 % of oxygen). He reported a reduction of sensitivity for all the octave tones from 64 to 8192 cycles, a reduction varying in amount from 0.8 db at 64 cycles to 10 db at 8192 cycles. However, these results are not to be taken at their face value, for they may be accounted for merely as instrumental changes. Rudmose and his associates (6) have shown that a telephone receiver when applied to the ear in a standard manner fails to maintain its normal output of sound pressure when there is a reduction in the density of the air. The changes that they observed were of the same order of magnitude as those in McFarland's experiment. This instrumental effect will account also for the earlier observations of Aggazzotti.

To the above evidence may be added the common reports of airplane pilots that no depreciations of hearing are evident to them even under conditions in which vision is seriously affected, and up to the point where a loss of consciousness puts an

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<sup>2</sup> The amount of reduction was stated in 'per cent of normal sensitivity,' which to us is unintelligible. The Western Electric 2-A audiometer, the instrument used in the tests, gives readings in discrete steps of 5 decibels relative to a standard ('normal') level. We should suppose that the authors meant 'decibels' rather than 'per cent' were it not that they reported steps smaller than 5.

end to observations. These indications are in harmony with all but Gelhorn and Spiesman's results in showing a high resistance of the auditory sense to anoxemia.

Another approach to this problem is the electrophysiological study of the ears of laboratory animals. Our method is to measure the electrical potentials generated in the cochlea during stimulation with sounds under normal conditions and in the course of oxygen deprivation. Let us first mention some preliminary observations that throw light on the problem.

Probably everyone who has made an extensive study of the cochlear potentials has found that when the ear is stimulated by a steady sound (of not too great an intensity) the potentials maintain a constant value over extended periods of time, and indeed as long as the physiological condition of the animal is unchanged. When for some reason, as perhaps a faulty use of anesthesia, the physiological condition deteriorates as shown by labored, infrequent respiration and light, irregular heart action the cochlear potentials become impaired. Their impairment appears only *in extremis*: when the physiological deterioration has proceeded to a serious stage; and we have come to recognize this cochlear sign as a warning of the impending loss of the animal.

A foregoing study (7) dealt in detail with the changes of cochlear potentials that result from the animal's death. These potentials suffer a serious impairment just before the heart stops, and thereafter continue to decline rapidly for several minutes. When they reach a value of perhaps 20 db below normal (depending somewhat upon the cause of death) they decline less rapidly, and so continue for a matter of hours until they are no longer discernible. No doubt a number of physiological conditions are involved during this course of changes, but it became evident that one of the important ones is oxygen starvation of the cells in which the potentials are generated. The present experiment clearly establishes this fact.

#### PROCEDURE

This investigation was carried out on 11 cats. The animals were first anesthetized with Dial (diallyl barbituric acid) injected intraperitoneally, and then were curarized (with Squibb's intocostin, by intravenous injection) to the point where reflexes were absent and respiration ceased. They were then maintained for the duration of the experiment by artificial respiration. It is important to note that these animals were in a state of minimal activity: the only remaining muscular exertion was that of the heart. The metabolic processes had only to sustain this action and a bare subsistence level of general cellular activity.

The inlet on the respirator was either open to the air—the 'normal' condition—or was attached by rubber tubing to a gas bag containing any desired respiratory mixture. The bag contained dilutions of air with nitrogen in varying amounts, giving oxygen compositions between 4 and 0.5 per cent. A gasometer was used to make up the gas mixtures, and it was placed in the respiratory line to indicate the rate of flow, which was kept at 1.5 liters/min. The heart action was regularly checked by listening with a stethoscope.

The cochlear potentials were picked up with an electrode on the membrane of the round window and were measured with a selective voltmeter (the General Radio

Type 736-A wave analyzer). The usual procedure consisted first of systematic measurements on stimulation with each of 12 frequencies from 100 to 15,000 cycles to ascertain the general condition of the ear. Then a 1000-cycle tone was adjusted to the intensity necessary to give a standard response, usually of 100  $\mu$ v., and thereafter it was steadily maintained at this level. Under normal conditions, as has already been indicated, this 100  $\mu$ v. response will continue with only minor variations for many hours and perhaps indefinitely.

The gas bag with its contents of diluted air was then connected to the inlet of the respirator, and observations of cochlear potentials were made at frequent inter-

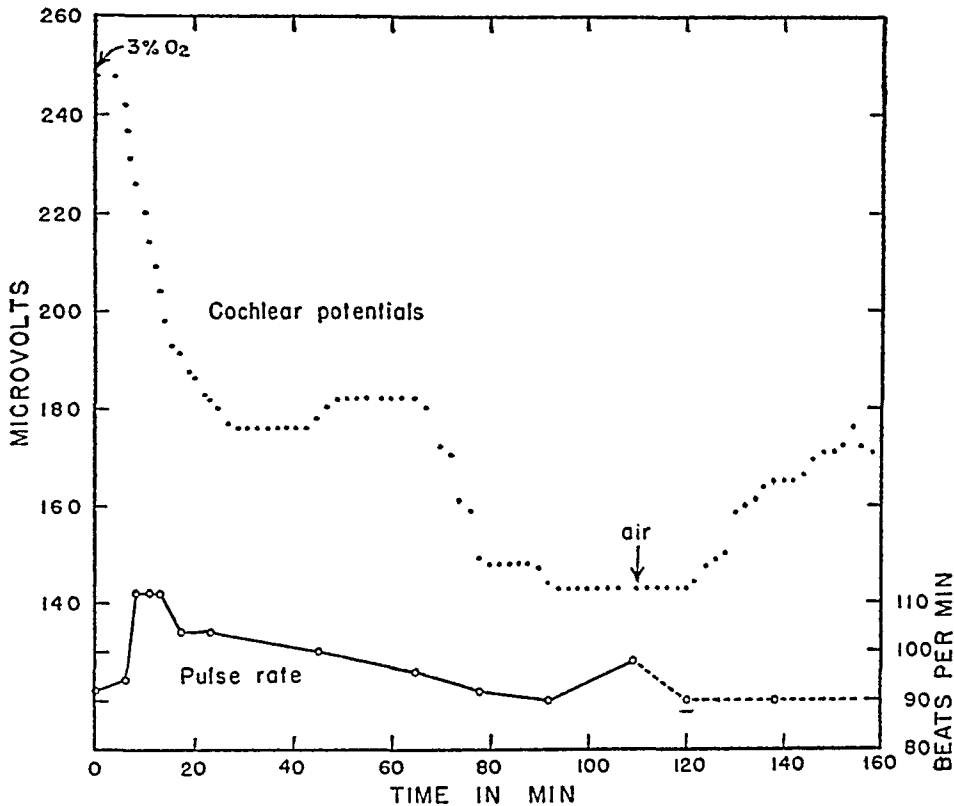


Fig. 1. EFFECTS ON COCHLEAR POTENTIALS of respiration with a mixture containing 3 per cent oxygen. The cochlear potentials are shown in microvolts by the series of points in the upper portion of the graph, and the pulse rate in beats /min. by the graph below. Arrows indicate when low-oxygen mixture was introduced and when air breathing was restored.

vals, usually every 15 sec. but sometimes oftener when changes were proceeding rapidly. Often the changes were followed to the point of death and somewhat beyond, but usually when the heart action became seriously impaired the gas bag was disconnected and air supplied to the respirator in the effort to resuscitate the animal. We have also used adrenalin, injected into the heart, and have succeeded sometimes in reviving an animal after the heart beat had become wholly imperceptible. After revival and a period of normal respiration, a further deprivation period ensued, and so on until the experiment was ended.

#### RESULTS

Our first observation was one already foreshadowed by the earlier discussion, that the cochlear potentials are notably resistant to anoxemia. Under the conditions

described, no changes were observed with gas mixtures containing more than 4 per cent of oxygen—mixtures equivalent to altitudes up to 40,000 ft. A little further dilution of the oxygen supply caused a slow, progressive deterioration of the potentials, until after an hour or two a level was reached from which there was little or no further decline. In such instances the animal evidently had made a physiological adjustment that sufficed, for a time at least, to maintain the cochlea without further deterioration at the level of function then reached.

A still further reduction of the oxygen supply caused a more marked effect, characterized by a rapid initial loss and then usually a leveling off. Figure 1 shows the results obtained on one animal with a gas mixture containing 3 per cent of oxygen. The cochlear potentials fell during the first 30 min. from an initial value of 248  $\mu$ v. to about 180  $\mu$ v. There they remained, with even a slight gain, for the next 40 min, reflecting a successful adjustment during this period. The adjustment could not be maintained longer, however, for the potentials then suffered a second rapid loss to a level around 145  $\mu$ v.

A study of the pulse record, shown in the lower curve of this figure, gives insight into the nature of the physiological adjustment. At the outset of the anoxemia the heart increased its rate of beating from 92 to 143 per min., which of course aided the interchange of oxygen between the blood and the bodily tissues. This high rate did not continue for long, but soon fell away, and after a time had sunk to a level too low to maintain the cochlear potentials at the 180  $\mu$ v. level. Then these potentials dropped to a lower level as shown.

After a period of oxygen deprivation of 110 min. the animal was respiration with air, whereupon, after a brief latent period, the cochlear potentials rose steadily to a new level around 175  $\mu$ v.

Figure 2 represents an animal that was exposed to an oxygen mixture of only 0.5 per cent. The pulse rate was rapid to begin with, and first declined, then rose and finally fell precipitously. Around 30 min. the heart action was noticeably irregular, with short periods of no beating, and when at 40 min. its complete failure seemed imminent the respiration was changed to air. This measure failed and the heart stopped around 43 min. During this time the cochlear potentials fell continuously from an initial value of 100  $\mu$ v. to a value, at the moment the heart stopped, of 1.3  $\mu$ v.

Figure 3 represents an experiment in which the impairment of cochlear potentials was carried almost as far as in the preceding, and yet an effort to resuscitate the animal was successful. The respiratory mixture contained 0.75 per cent of oxygen, and in the first trial was given for 40 min. At the end of this time the heart action was weak and irregular, and the cochlear potentials had fallen from 105 to 6.5  $\mu$ v. Respiration with air strengthened the heart beat without changing its rate and raised the cochlear potentials to 27  $\mu$ v. A second trial with the 0.75 per cent oxygen mixture caused a rapid deterioration of heart action and reduced the cochlear potentials further to 1.2  $\mu$ v. The introduction of air at this point was too late, for though the heart action improved for a time it then weakened and ceased. The cochlear potentials showed a rise to 5.2  $\mu$ v. and then a fall. It is typical that this fall of potentials began a little while before the heart action showed any turn

for the worse, and that it underwent no particular change in its course at the moment of the heart failure.

It is possible, as figure 4 shows, to carry an animal through several periods of anoxemia, provided that each exposure is brief and the heart is not allowed to deteriorate too far. The animal represented here was exposed to a mixture containing 0.5 per cent of oxygen for three periods of about 10 min. each in quick succession and then after an hour of air breathing was given another period of exposure. Each period of oxygen deprivation caused a loss of cochlear potentials and each period on air gave a partial recovery of these potentials. The fourth exposure to the low-oxygen mixture caused the heart to fail badly and for a period of two minutes, just after air respiration was started, its beat could not be detected. Adrenalin was injected into the heart, and in two minutes more the pulse was strong and regular and the cochlear potentials had recovered markedly. A second injection of adrenalin was made to strengthen the heart further, and then this animal's ears were preserved for histological study by an intravital injection of fixing fluid.

It is clear from the results represented here that the cochlear potentials suffer from oxygen deprivation when this deprivation is extreme, and recover somewhat when adequate respiration is restored. Additional periods of deprivation cause further losses, and each again is followed by a partial recovery when air breathing is instituted. There is nearly always a net loss of cochlear potentials from each period of exposure, even though the animal may survive and judging from the character of its heart action may seem little the worse on account of its treatment. Yet the recovery of cochlear potentials is always limited, and a normal level is not regained even after protracted air breathing. As figure 4 shows, after the third exposure and the re-establishment of air breathing the level of potentials reached after 4 min. was not appreciably

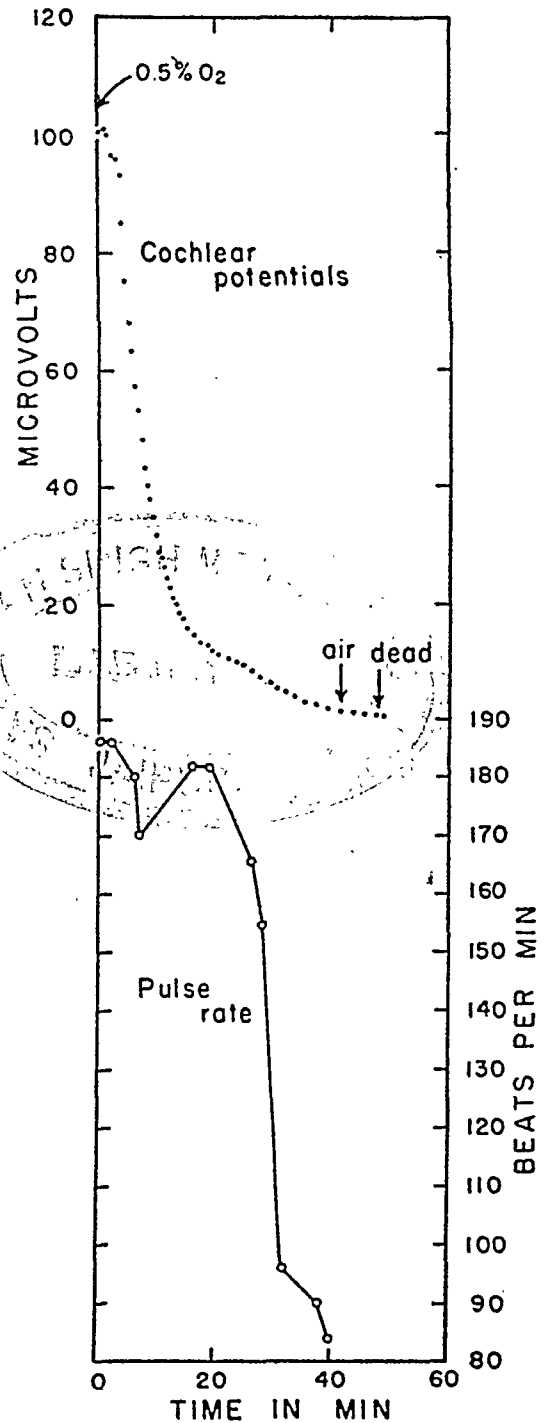


Fig. 2. EFFECTS of a respiratory mixture containing 0.5 per cent oxygen.

improved by as much as an hour's continuation of the air breathing. It is necessary to point out that this study has dealt only with acute conditions, and it remains to be discovered whether longer periods of time, or days or weeks, would restore the response further.

The results given so far were obtained with a single stimulus tone of 1000 cycles, and the question naturally arises whether all tones would present this same pattern of changes. McFarland considered this matter in his audiometric study, and believed that he had demonstrated a frequency relation—a greater susceptibility to loss in the higher range—but as we have seen his results are brought into question by the possibility of purely instrumental variations. The following experiment dealt with this aspect of the problem.

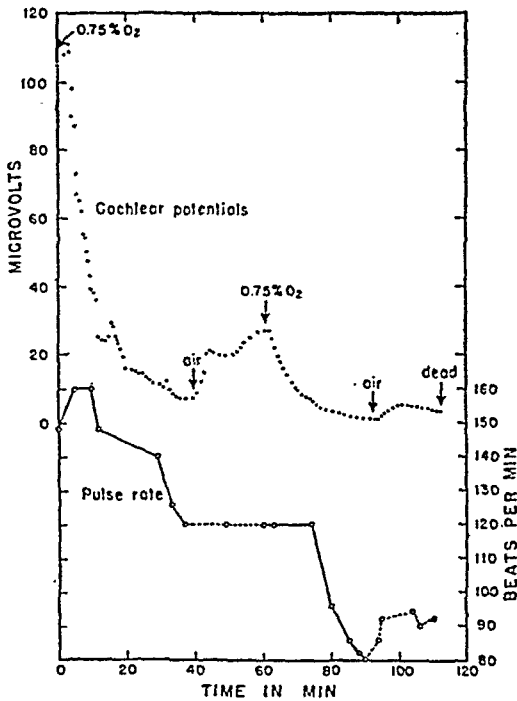


Fig. 3. EFFECTS of two exposures to a respiratory mixture containing 0.75 per cent oxygen.

In one animal, represented in figure 5, a normal sensitivity curve was obtained before any anoxemia was introduced. This was done by ascertaining, for 12 different tones over the range from 100 to 15,000 cycles, the stimulus intensity necessary to produce a standard magnitude of cochlear potentials (a response of  $1 \mu\text{v.}$ ). The results are given in the lowermost curve of the figure, and show about the usual sensitivity function for the cat, with the keenest sensitivity for the middle and medium high tones. Then the animal was exposed to a mixture of 0.5 per cent of oxygen for 30 min., by which time the cochlear potentials had passed through the rapid phase of deterioration and were falling only slowly. The curve closely resembles that shown in figure 2, except that it leveled off sooner and at 30 min. was declining even more slowly. At this time a second set of sensitivity measurements was made,

again by presenting the various tones at the intensities necessary to produce the standard response. These measurements took 8 min. to carry out, and the results are of course subject to error on account of the progressive deterioration undergone during that time. We have sought to correct for this error by measuring the 1000-cycle response at the beginning and end of this testing period and adjusting the readings on the assumption that the deterioration thus shown was uniformly distributed over the period. The upright triangles in figure 5 represent the uncorrected readings and the inverted triangles the corrected readings. The corrected curve closely follows the form of the normal curve, with a mean difference of 42.6 db and a mean variation of 1.3 db. Hence it seems that the deterioration of cochlear potentials due to anoxemia is independent of frequency.

## DISCUSSION

As an examination of the first four figures will show, there were large variations in the temporal relations between changes in the oxygen supply and the resulting changes in the magnitude of cochlear potentials. When a respiratory switch was made the cochlear potentials sometimes began to show an effect after only a brief interval of 30 to 90 sec., and at other times this latency was as great as 8 min. The variations largely reflected the circulatory conditions then prevailing: the rate and

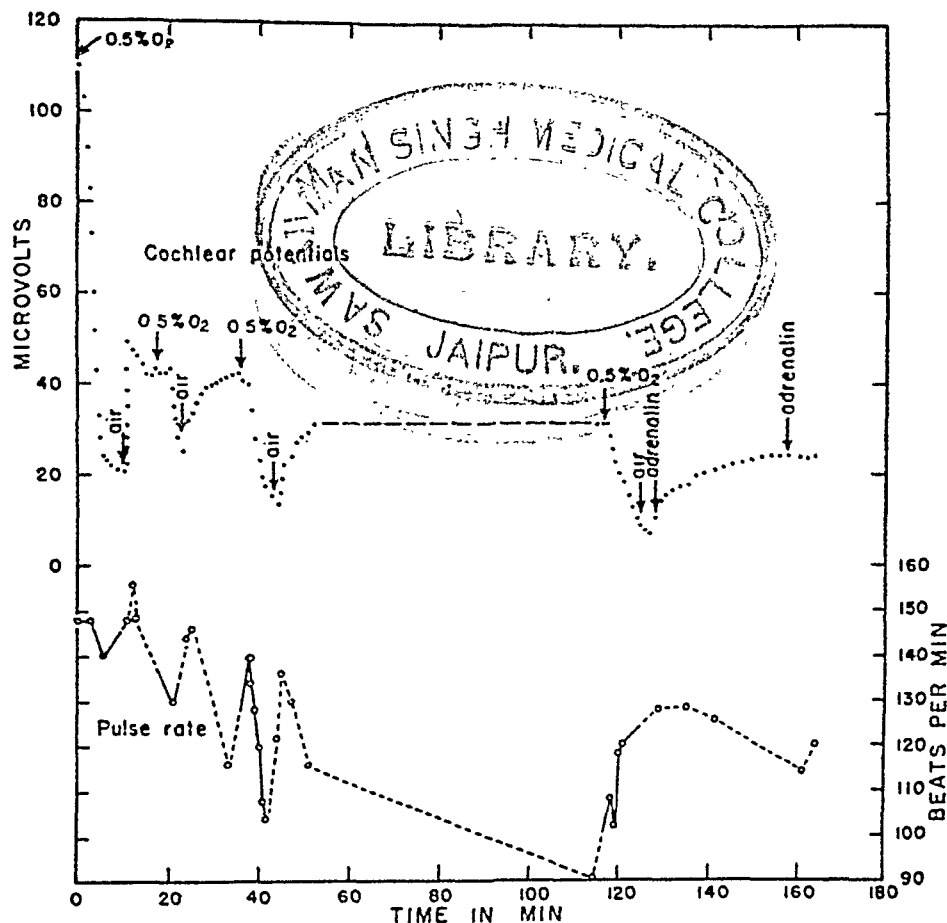


Fig. 4. EFFECTS OF REPEATED EXPOSURES to a mixture containing 0.5 per cent of oxygen. Near the end of the experiment, adrenalin was administered to facilitate heart action.

strength of the pulse, as we could observe, and no doubt also the dilatation of the capillaries and the degree of utilization of oxygen by the cochlear cells.

It is necessary at this point to consider the specific seat of the potential changes resulting from anoxemia. There is no doubt that these changes take place in the hair cells of the organ of Corti. Many lines of evidence—especially from examination of albinotic and other animals with malformed or atrophic cochlear structures and from experiments on stimulation deafness—point to these cells as responsible for the generation of the cochlear potentials.

If this site of the anoxic changes is the correct one, we are presented with a peculiar problem in view of the mode of nourishment of the hair cells. As is well known, these cells, along with the entire organ of Corti, do not possess any direct

blood supply. They evidently derive their nourishment from the endolymph, which, as Corti first suggested, is probably supplied by the stria vascularis, which is a band of pigmented cells lining the outer wall of the cochlear duct and richly served with blood vessels. The mean distance of this band from the outer hair cells (specifically, the distance from the middle of the stria to the middle row of hair cells) was measured in one cat and found to vary from about 0.20 mm. at the apical end of the cochlea to about 0.53 mm. at the basal end, and throughout the middle portion of the cochlea had a fairly consistent value of 0.33 mm.

Let us consider the structural relations in further detail. The hair cells are directly exposed to the endolymph only at their outer, ciliated ends. In addition, the

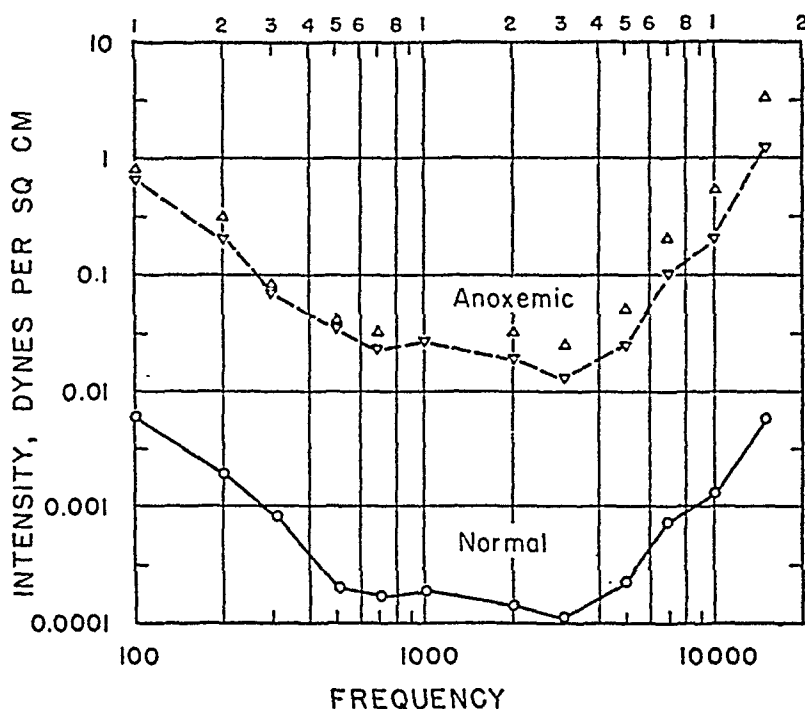


Fig. 5. ANOXEMIC EFFECTS as a function of stimulus frequency. Curves show the stimulus intensity required at various frequencies to produce a standard response of 1 microvolt, under normal conditions and in severe anoxemia.

outer hair cells have their midportions suspended in the fluid of Nuel's space, but this fluid, though common to the inner and outer tunnel spaces, does not communicate freely with the endolymph. The inner hair cells are closely surrounded by the phalangeal cells and the border cells. Hence it appears that oxygen transported by the red blood cells diffuses from the stria vascularis over a mean path of about 0.33 mm. across the cochlear duct, and then enters the hair cells directly through their ends or more circuitously through their side walls after penetrating the reticular membrane and the further intervening fluid or cells.

We need to judge as to the reasonableness of this conception of the nourishment of the hair cells in view of the latencies of cochlear potential changes observed in this experiment. Our observations are not ideal for this purpose, for we might better relate the potential changes to the oxygen content of the blood rather than to the respiratory supply. Still, these results provide the basis for a preliminary view.

Our minimal latencies, as stated, were around 30 sec., but these times include

certain external conditions. We must allow about 13 sec. for the gas entering the respirator to pass along the length of tubing leading to the animal, and some further time, which is difficult to estimate, for the replacement of the alveolar air. Then there is the time of the circulatory movement to the cochlea. These peripheral conditions will consume more than half of the observed time, and will leave something of the order of 10 to 12 sec. for the diffusion in the cochlea. This amount of time appears sufficient for the process. Yet there is no great excess of time, and we have to conclude that the hair cells do not have at their command any appreciable reserve of oxygen but must be furnished with a continual supply. Their needs though small are steadily demanding.

A comparison of the present results with those obtained earlier in the study of the death function reveals close similarities in the amount and temporal course of the cochlear potential changes, and indicates that the principal effect of death on these potentials is the stopping of the oxygen supply. We therefore feel warranted in carrying over to the present problem a principle established in the death study and not examined further here, namely, that the rate of deterioration of the cochlear potentials under these adverse conditions is unaffected by the intensity of stimulation (provided of course that this intensity is held below the level of physical injury). We have limited evidence here for still another relation found in the death study, that despite the deterioration of the responses the form of their intensity function is unaltered: their magnitude maintains its linear relation to the sound pressure at low and intermediate levels of stimulation and its distorted relation at high levels. Hence we conclude now, as we did in contemplation of the nature of cochlear activity after death, that the cellular processes concerned in the generation of the cochlear potentials are essentially simple in nature, and consist of a transformation of the mechanical energy of the stimulus into electrical energy and do not involve any liberation of stored energy.

We conceive that certain processes of a metabolic nature, dependent upon a continual supply of oxygen, provide a basis for this transformation but do not enter into it directly. Probably these processes have the duty of maintaining a base value of electric polarization in the hair cells. This polarization may be thought of as a surface positivity of the cell membrane. It is likely that under normal conditions, in the absence of stimulation, it is held at a fairly constant level and its average value still remains unchanged for ordinary stimulation. However, under the influence of sounds its instantaneous value changes, or at least its external influence changes, so that in the neighborhood of the cells there are electric fluctuations that are a faithful copy of the mechanical pressures. These fluctuations are the cochlear potentials.

Now when the oxygen supply is drastically curtailed the base polarization becomes reduced and in many cells is wiped out altogether. The sensitivity is therefore impaired. The cells whose polarization is lost of course no longer make their usual contributions to the electrical responses. The cells in which some polarization is still present will deliver their usual output as long as the stimulus is weak and the fluctuations do not exceed the polarization potential, but when the stimulus intensity is raised and the fluctuations are greater the output is impaired. Hence the response as a whole maintains its linearity only at the lowest response level and quickly grows distorted as this level is raised.

When the period of anoxemia is ended and normal respiration is restored the base polarization returns in some of the cells but remains absent or well below normal in many others. Therefore we find only a partial recovery of sensitivity.

We have made a histological study of the two ears of a cat that was exposed to extreme anoxemia over a period of 4 hours, and which during this time underwent a particularly severe loss of cochlear potentials. The animal survived until it was injected for the histological treatment. Microscopical examination of sections of the ear disclosed marked pathological changes in the organ of Corti over the whole length of the cochlea. The sensory structure had largely lost its differentiated character and was reduced to a misshapen mass in which cell nuclei were present but no cell boundaries could be made out. The arch of Corti could be seen, though it was greatly modified in form. No hair cells could be surely identified, though certain nuclei were present that may have belonged to the inner hair cells. On the other hand, the internal sulcus cells and the stria vascularis remained in fairly good condition. Reissner's membrane was greatly ballooned out and through most of the cochlea was closely adherent to the wall of the scala vestibuli. These changes fully account for the observed impairment of electrical responses.

#### SUMMARY AND CONCLUSIONS

Measurements of the electrical potentials of the cochlea carried out in cats have revealed deleterious effects upon the ear as a result of oxygen deprivation. The deprivation has to be severe and is produced by respirating with air containing less than 4 per cent of oxygen—a mixture that gives the same partial pressure of oxygen as that of the atmosphere at an altitude exceeding 40,000 ft. above sea level. As is well known, this degree of oxygen lack cannot be endured by man without loss of consciousness and even of life itself; and it was possible for the cats only because they were maintained at minimal activity under anesthesia and curare and were artificially respired.

As the anoxemia develops over several minutes the cochlear potentials undergo a rapid initial loss and then tend to level off. With extreme deprivations, which carry the animal close to the point of death, the losses may amount to 40 db and more. On a return to normal air breathing the responses recover, but only partially. Repeated exposures to anoxemia give a cumulative deterioration of the responses. The effects are found for all tones, without appreciable variation with frequency.

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# SITE OF ACTION IN THE CENTRAL NERVOUS SYSTEM OF A BACTERIAL PYROGEN

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NUMEROUS investigators have confirmed and extended the observation of Isenschmid and Schnitzler (1) that the hypothalamus is essential for regulation of body temperature. Notable work in this field was that of Ranson and his co-workers (2) who clearly separated the heat loss center from the heat conservation center in the hypothalamus. It followed logically that fever result from disturbances of the hypothalamic thermoregulatory mechanism. Ranson, Clark and Magoun (3) tested this hypothesis experimentally but were unable to draw definite conclusions regarding the rôle of the hypothalamus in pyrogenic fever. The hypothesis was further weakened by results of Haertig and Masserman (4) who found that cats with massive hypothalamic lesions and impaired thermoregulatory ability developed fevers of infectious origin.

Recently a purified and relatively non-toxic pyrogenic extract of a *Pseudomoma* species that gives dependable febrile responses (5) was made available as a tool to re-investigate this problem. Efforts were made first to determine whether the pyrogenic response is mediated through the nervous system and, having established this point, to locate the level of neural integration of the pyrogenic response within the neuraxis.

## MATERIAL AND METHODS

Dogs and cats were used. The pyrogenic extract, known as Pyromen (5), was usually employed but in several instances other pyrogens were tried. Dosages ranged from 20 to 200  $\mu\text{g}/\text{kg}$ . of body weight. Intraperitoneal and intravenous routes of administration were employed in the cat; the intravenous route was used in the dog. Control runs were made with intravenous injections of pyrogen-free saline solution in many of the experiments. Rectal temperature was recorded continuously with a resistance thermometer on the Leeds and Northrop Micromax potentiometer in most animals. In other animals periodic rectal temperatures were determined with a clinical thermometer. In some experiments cutaneous and rectal temperatures were obtained with iron-constantin thermocouples and a potentiometer. Wherever temperature regulation was impaired as a consequence of neurological lesions, environmental temperature and humidity were controlled in a specially constructed room. In each experiment an attempt was made to obtain a balanced control period prior to injection of the pyrogen. In the absence of febrile responses animals were reinjected one or more times.

White blood cell counts were made in a few experiments. In several trials sodium salicylate and pentobarbital were employed in an attempt to break the fever. Atropine was used to prevent the gastrointestinal side reactions in one spinal dog. Vascular changes occurring in the rabbit's ear were studied in cooperation with Dr. R. G. Williams by means of the preformed tissue chamber.

Neurological lesions were made under aseptic conditions and with ether or pentobarbital anesthesia. Transections of the exposed spinal cord were performed with a sharp scalpel at various levels. Decortication was performed with a scoop. Massive bilateral thalamic and caudal hypothalamic

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lesions were placed in 5 cats with the Horsley-Clark stereotaxic instrument.<sup>2</sup> Decerebration with no attempt at asepsis was performed in 2 cats by the Sherrington scoop method, in 9 cats by the anemic method of Pollock and Davis (6) and in 8 cats by the Schmidt method (7). Chronic as well as acute preparations were studied, except in the case of the decerebrated animals which were studied during the first 48 hours following surgery. All neurological lesions were confirmed at autopsy. Spinal cord transections were verified by silver-stained serial sections through the region of the lesion. The extent of thalamic and hypothalamic lesions was determined on serial sections stained by the Weil technique. The level of anemic decerebration was established by gross inspection of the brain for the distribution of dye following injection of an aqueous solution of methylene blue under high pressure into the aorta.

## RESULTS

*Normal Animals.* One hundred thirty trials were carried out on 23 cats. The response to administration of pyrogenic substances, while variable and occasionally absent, consisted typically of the following sequence of events: drowsiness, rising rectal temperature and falling skin temperature of the ears, nose and footpads during

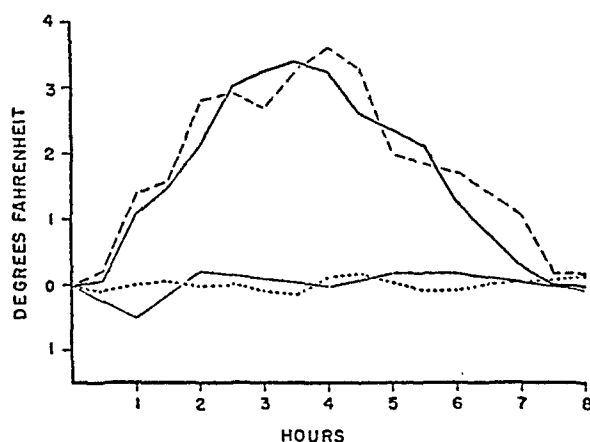


Fig. 1. CONTINUOUS RECORDS of rectal temperatures. The solid heavy line represents a typical response to an injection of Pyromen in an intact cat; the solid thin line represents the response to an injection of pyrogen-free saline solution in an intact cat; the dotted line represents the response to an injection of Pyromen in a cat 30 days after transection of the lower cervical spinal cord; the broken line is the febrile curve of a cat on the ninth day following massive bilateral caudal hypothalamic destruction. Injection was given at zero on the abscissa scale after the animals had been stabilized with respect to their rectal temperature.

the first 15 minutes, piloerection and moderate shivering during the second half hour. By the end of the first hour the febrile response averaged  $2^{\circ}\text{F.}$ , with drowsiness and piloerection continuing but shivering ceasing. The fastigium was attained by 4 hours and averaged  $3.6^{\circ}\text{F.}$  at which time piloerection ceased. Defervescence then set in with a return of the temperature curve to the baseline by 8 hours (fig. 1). Occasionally fever occurred in the absence of overt shivering and piloerection. Retching, vomiting, defecation and urination occurred in some animals during the second half hour. Respiratory changes, chiefly in the direction of a rate increase, frequently occurred during the early period. Animals failing to yield a fever were given additional injections of the pyrogenic substances until the febrile response was obtained.

The pyrogenic response in the dog was qualitatively and quantitatively similar to that of the cat with several exceptions. The dog invariably responded to pyrogenic drugs with a fever (80 trials in 30 animals). The side reactions of vomiting, defecation and urination usually occurred; piloerection was much less pronounced and respiratory changes were more constant than in the cat.

<sup>2</sup> We are indebted to Prof. H. W. Magoun, of Northwestern University, for preparation of these animals.

*Spinal Cord Transection.* The spinal cord of one dog was transected at T-5, another dog at T-9, and a cat at L-1. These 3 animals yielded typical pyrogenic responses a day or two postoperatively when Pyromen was administered.

Transections of the spinal cord were prepared at C-7 or C-8 in 3 cats, at T-1 and T-2 in 2 cats and at C-7 in a dog. The cats were studied at various times from 5 to 30 days postoperatively; the dog was studied for 60 days postoperatively. These animals showed no appreciable ability to maintain a normal body temperature and had to be kept in a warm room. Even though the environmental conditions were maintained constant, each of these animals showed a slow cyclical variation in rectal temperature with elevations as great as  $3^{\circ}\text{F}$ . (apparently related to food intake). Twenty-one injections of Pyromen<sup>3</sup>, two injections of a similarly purified typhoid extract<sup>3</sup> and one injection of 35 mg. of pyrexin<sup>4</sup> failed to elicit a single unequivocal febrile response. *Cat 21* was tested 4 times with Pyromen and typhoid extract over a 30-day period and *dog 5* was tested 8 times with all three pyrogenic materials over a postoperative period of 60 days. In spite of the failure of pyrogens to elicit a febrile response in these high spinal animals, the cats occasionally had the usual side reactions. Retching, vomiting, defecation, urination, drowsiness and respiratory changes occurred in the dog during each trial in which pyrogens alone were given, but the gastrointestinal and urinary vesical changes were blocked by atropine whenever this drug was administered prior to the pyrogen. Slight reductions in skin temperature occurred in the dog but these appeared to be part of the visceral component of the mass reflex; subsequent prevention of skin temperature changes by atropine, which also blocked defecation and hence the mass reflex, bore out this impression. At no time was shivering seen caudal to the level of the lesion.

A marked leucopenia affecting all white cell types occurred in the dog with the cervical spinal cord sectioned, even though there was no febrile response. Leucopenia followed by leucocytosis during both pyrogenic and physical fever has been reported by other investigators (8, 9). We have seen these changes in intact animals and in man. Studies employing the preformed tissue chamber in the rabbit ear revealed intense vascular spasm during the chill phase after systemic administration of Pyromen. It was possible to observe the absence of adhesion of leucocytes to the cutaneous blood vessel walls; therefore such a process is believed not to have been a factor in the leucopenia. The findings in the cervical spinal dog appear to dissociate the peripheral blood cell changes from the febrile response.

*Decortication and Massive Thalamic Destruction.* One decorticate cat had only the pyriform area of the cerebrum intact and the basal ganglia were completely destroyed. This animal responded to injections of Pyromen with the usual febrile response as early as one day postoperatively. Another cat, with massive bilateral thalamic lesions, was tested four times from the 4th to the 10th postoperative day and exhibited a fever on each occasion.

*Massive Bilateral Caudal Hypothalamic Lesions.* Four cats were studied. A febrile response was seen as early as one day postoperatively and 2 animals showed re-

<sup>3</sup> Both supplied by Dr. N. M. Nessel of the Baxter Laboratories, Morton Grove, Ill.

<sup>4</sup> Kindly provided by Dr. Valy Menkin of Temple University.

sponses as late as the 25th postoperative day when the experiments were terminated. Sixteen trials were made. There were 13 definite febrile responses and only 3 equivocal responses. In no instance was vomiting, urination and defecation seen and in only one trial was there a suggestion of shivering and piloerection (fig. 1). These animals were all relatively poikilothermic.

**Decerebration.** Nine cats were decerebrated by the Schmidt technique and tested with Pyromen immediately after operation. Decerebrate rigidity was marked but the animals tended to lose heat rapidly at an environmental temperature of 85° F. The dorsal transection level was through the superior colliculus and the ventral level varied. In the animals in which the ventral level traversed the rostral part of the midbrain (7 in all) no febrile response was elicited with a single injection of Pyromen and the rectal temperatures continued to drop. The usual side reactions were present

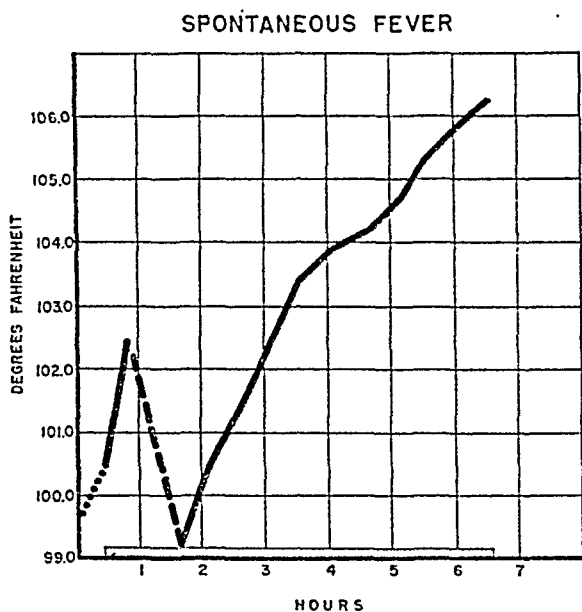


Fig. 2. CONTINUOUS RECORDING of the rectal temperature of anemically decerebrated cat 5 with a spontaneous fever. One hour after decerebration the rectal temperature was below 100° F. and radiant heat was applied (dotted line). Shivering set in (stippled bar) and the lamp was removed. When the rectal temperature had reached 102.5° F. the animal was placed in a refrigerator at 36° F. for 40 minutes (broken line). Upon removal from the refrigerator (solid line) the animal continued to shiver and the rectal temperature soared to 106.3° F., at which point respiratory failure occurred.

and severe respiratory embarrassment was encountered in all. This led to premature death in some instances as mucus plugged the tracheal canula and/or lung edema developed. In one cat with the transection passing just in front of the mammillary bodies a marked febrile response occurred; violent shivering persisted until death 80 minutes after the injection of Pyromen and at that time the rectal temperature had risen to 107.8° F. In the 9th animal the level of the transection was at the rostral border of the pons. This animal developed a spontaneous fever which was unaffected by a near surgical dose of pentobarbital. Prior to administering the pentobarbital, tonic and clonic convulsions were seen. The footpads were cool from the time of operation and throughout the experiment and shivering did not occur.

Neither of the 2 cats decerebrated by the scoop method and injected with Pyromen responded with a fever. They showed rapid declines in rectal temperature at an environmental temperature of 85° F.

Nine cats were decerebrated by the anemic method and studied immediately after surgery. The ventral level of ischemia varied from the rostral border of the

medulla oblongata to the rostral one-third of the pons. These preparations showed marked decerebrate rigidity and remarkable ability to maintain their rectal temperature at environmental temperatures as low as 76° F. Two developed spontaneous fevers (fig.) 2. Four responded with fevers upon injection of Pyromen (figs. 3 and 4). Three gave only questionable pyrogenic responses. The 2 animals developing spontaneous fevers began to shiver and displayed peripheral vasoconstriction soon after surgery. Both had hyperpyrexia at the time of death and autopsy revealed considerable hemorrhage into the brain stem in the region of the ligature on the basilar artery. The unequivocal pyrogenic responses were characterized by the usual latency and drop in skin temperature but the animals showed more marked shivering and more constant and violent side reactions than were seen in intact animals. These experiments, because of premature respiratory failure, were of too short duration to

## FEBRILE RESPONSE TO PYROGEN

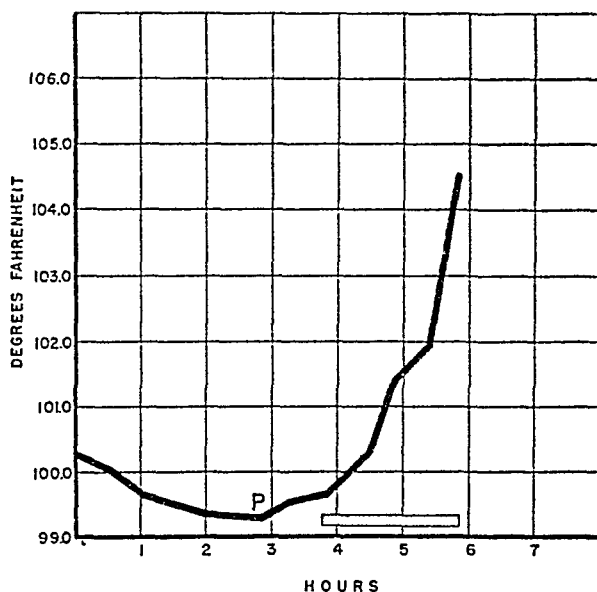


Fig. 3. CONTINUOUS RECORDING of the rectal temperature of anemically decerebrate cat 2. The recording began 20 minutes after termination of surgery. The Pyromen solution was injected at P. The stippled bar indicates the presence of shivering.

permit evaluation of the defervescence stage of the pyrogenic response. Decerebrate cat 24 showed stepwise increases in rectal temperature following each successive injection of Pyromen (fig. 4). In decerebrate cat 2, two intravenous injections of 0.5 gm. each of sodium salicylate failed to reduce the rectal temperature.

## DISCUSSION

The fever that occurs in a pyrogenic response is the result of increased heat production and reduced heat loss. The former appears to be due chiefly to an increase in skeletal muscle metabolism. In many of our experiments this was apparent in overt shivering. In other experiments overt shivering was not observed but an imperceptible increase in muscle tone, as shown by Burton and Bronk (10), may have been present and could have increased heat production. It is probable that some epinephrine is secreted during the chill phase of the fever and its calorogenic effect may have contributed to increased heat production. An additional source of body heat is provided by the increased metabolism that results from an increase in body temperature.

Reduced heat loss is brought about chiefly by cutaneous vasoconstriction and piloerection, the latter occurring particularly in the cat. Cutaneous vasoconstriction produces a reduction in skin temperature (11-14) that results in an appreciable layer of surface insulation, augmented further by the dead air spaces formed among the erected hairs.

The effectors involved in increased heat production obtain their innervation from the ventral horn neurons of the spinal cord and the cranial motor neurons to striated muscle. The latter, however, comprise but a small proportion of the total motor units. The smooth muscle of blood vessels and hair follicles receive their nerve supply from postganglionic neurons present in the sympathetic chain ganglia and indirectly from the intermediolateral cell column in the thoracic and upper lumbar spinal cord. This latter region also supplies some nerve fibers directly to the adrenal medulla. The main sensory inflow as well as the main motor outflow involved in fever

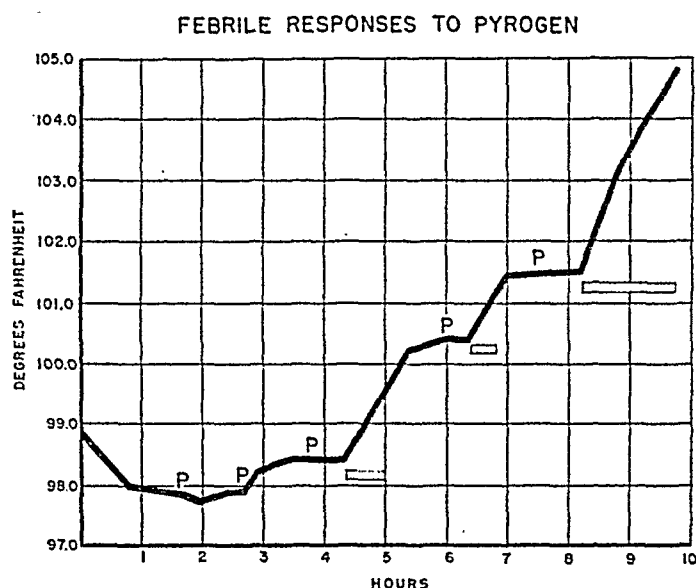


Fig. 4. CONTINUOUS RECORDING of the rectal temperature of anemically decerebrated *cat 24*. Injections of Pyrogen were given repeatedly at P. Shivering is indicated by the stippled bar.

is intact in an animal whose spinal cord is functionally separated from the brain stem at the lower cervical level. In view of consistent failure to obtain febrile responses from such preparations, even as late as two months postoperatively (a period far in excess of that necessary to insure recovery from spinal shock), it is concluded that the pyrogen acted neither on the spinal mechanism directly nor peripherally. This is further borne out by the absence of shivering caudal to the level of the lesion, the failure of the pyrogen to provoke piloerection and, finally, its failure to produce a decline in skin temperature when the mass reflex was abolished by atropine. The results obtained in animals with the spinal cord transected at midthoracic and lower levels, which gave febrile responses as early as two hours postoperatively, indicate that only part of the thoracic cord need be in functional continuity with higher nerve centers to produce fever. They strengthen the view that operative trauma and spinal shock did not seriously interfere with pyrogenic responses.

The remaining possibility, then, is that the pyrogen stimulated one or more co-ordinating centers in the brain and that these centers were required to be in functional

connection with the spinal and peripheral mechanisms. Decortication, massive thalamic and hypothalamic destruction and decerebration did not abolish the pyrogenic response. Since the lowest level of decerebration was at the rostral border of the medulla oblongata, at least the essential coordinating mechanism in the production of fever exists in the medulla oblongata and/or cervical spinal cord. Although the possibility of a center in the cervical spinal cord was not eliminated experimentally, one would suspect that the medulla oblongata contains the responsible integrating mechanism.

In so far as could be determined, destruction of most of the cerebral cortex, basal ganglia and thalamus did not alter any aspect of the pyrogenic response. The animals with massive hypothalamic damage and seriously impaired thermoregulatory ability, however, differed from intact animals; they did not shiver, piloerect, vomit or defecate (except in one experiment during which equivocal shivering and piloerection were noted). In the one Schmidt decerebrate preparation in which the caudal hypothalamus was intact a typical and exaggerated pyrogenic response took place.

The Schmidt and scoop decerebrate preparations with all or most of the midbrain intact never showed fever, shivering, piloerection or cooling of the skin although they did have the side reactions. In the large majority of animals with the level of decerebration at pontile or medullary levels pyrogenic or spontaneous fevers occurred. Shivering and cutaneous vasoconstriction were exaggerated and markedly protracted and the side reactions were consistently present and were severe. However piloerection appeared somewhat diminished. Another important difference was noted, namely, that at no time was a definite defervescence stage seen; the records often suggested that, had these animals lived long enough, extreme degrees of fever might well have occurred.

The pontile and medullary animals were prepared by the anemic method but the neurological signs and results of intra-aortic injection of dye under high pressure justify the assumption that the level of transection was as precise and as complete as if it had been made with a knife. The results of experiments in the one Schmidt preparation with complete anatomical transection extending from rostral border of the pons to the superior colliculus and with no midbrain tegmentum present, further support the conclusion that the pontile animal is able to have a coordinated febrile response. Furthermore, although the pontile animals were not specifically tested for their ability to thermoregulate, it was evident that they could maintain their rectal temperature in a cool environment more effectively than midbrain animals. These results suggest that there exists in the tegmentum of the midbrain a center that tends to inhibit shivering, vasoconstriction and the febrile response itself in the absence of the caudal hypothalamus.

In the experiments of Haertig and Masserman (4) infectious fevers occurred in cats that had massive hypothalamic lesions and were relatively poikilothermic. The lesions did not involve the caudal hypothalamus. Ranson, Clark and Magoun (3) prepared 21 cats with hypothalamic lesions and tested them with typhoid-paratyphoid vaccine. One of 6 cats with lesions in the caudolateral part of the hypothalamus responded with a good fever, and 6 showed marked falls in rectal temperature. Fifteen cats had more rostral hypothalamic lesions. Ten of these failed to show any

fever and the remaining 5 responded with only a very slight fever. Furthermore, their data demonstrated that there was a component in the vaccine which produced hypothermia. The pyrogenic material used in our experiments contains no factors that could be demonstrated to produce hypothermia.

#### SUMMARY

The response to injection of a purified pyrogenic extract of a *Pseudomonas* species was studied in cats and dogs, both intact and with central nervous system lesions.

Transection of the lower cervical and upper thoracic spinal cord in acute and chronic animals prevented the febrile response but not the side reactions. Cutaneous vasoconstriction and piloerection as well as shivering below the lesion did not occur. Transection of the spinal cord at or below T-5 did not prevent the development of fever. It was concluded that the febrile response to the pyrogen was mediated through the central nervous system and that the spinal cord needed to be in functional communication with one or more centers in the brain.

Decortication, thalamic and caudal hypothalamic lesions did not prevent the febrile response. Decerebrate preparations in which most of the midbrain remained intact failed to show a febrile response. However animals decerebrated at a pontile or medullary level had pyrogenic and spontaneous fevers. It appears that an integrating mechanism exists in the medulla oblongata and/or upper spinal cord which, when connected with the spinal cord, below the cervical region, was fully capable of evoking a febrile response to a pyrogen and in some instances of initiating 'spontaneous' fever.

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# RELATION BETWEEN SPIKE HEIGHT AND POLARIZING CURRENT IN SINGLE MEDULLATED NERVE FIBERS<sup>1</sup>

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THE effect of polarization on the height of the action potential (the magnitude of the 'alteration') in multifibered preparations has been the subject of many investigations, some dating back to the middle of the last century.<sup>2</sup> One of the most recent of these is included in Lorente's *A Study of Nerve Physiology* (2). In that monograph occur these statements: 1) "In the analysis of the problem" of electrotonic potentials "fiber diameter will eventually have to be taken into consideration;" and 2) "In the analysis of the problem that is possible at present the use of the single fiber approximation is justifiable." Some qualitative observations on the effect of polarization on the action potential of single medullated fibers were made in this laboratory in 1934 (3). The present investigation, an attempt to ascertain quantitatively the effect of polarization on the height of the spike of single fibers, was begun in 1943 and, after interruption by the war, was resumed in June 1947. A preliminary report of a part of the work was made in 1948 (4).

## METHODS

The single fiber preparation has been mainly the sciatic nerve of the green frog together with the branch extending to the tip of the 3rd digit (the phalangeal or toe nerve preparation) (5). In about one out of three of these preparations there is a fiber of such outstanding excitability that it alone responds at the lead from the toe nerve to stimulation of the parent trunk with shocks that attain the threshold of that fiber. Twice only have we succeeded in obtaining comparable and adequate preparations by splitting the unbranched part of the sciatic nerve. The action potentials were recorded with the cathode ray oscillograph after passage through a resistance-capacity coupled amplifier. The networks used are shown in figure 1. Network A was the most usual one. Circuit C has certain disadvantages from the standpoint of the present investigation: when the anode lies between the stimulated locus and the lead it often blocks the fiber whose spike is being recorded, before the full range of the cathode effect has been achieved. This difficulty is minimized, however, by the fact that during cathode polarization the anode is on a thicker part of the nerve. An additional disadvantage is that it supplies conditions favorable to restimulation of the fiber beyond a block at the anode (6). The electrodes were of the calomel half-cell

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<sup>2</sup> A review of the early literature will be found in Schmitz and Schaefer (1).

type. The strength of the applied current is expressed in arbitrary potentiometric units and in many of the observations on single fibers the limiting strengths employed were those producing anode or cathode block or deformation of the spike. The linearity of the potential divider was tested by passing current through it in series with a dead sciatic nerve. The amplifier was checked for linearity of output.

The usual procedure has consisted of mounting the nerve vertically in the moist chamber (fig. 1) immediately after the dissection. If it seemed to contain a fiber of outstanding excitability it was then dripped slowly with Ringer's solution for some time while observing the spike elicited by threshold stimuli delivered at the rate of about one per second. Having thus obtained reasonable assurance that but a single fiber was responding, observations were begun. Often, however, the nerves were left in Ringer's solution for an hour or longer, some at room temperature, others at about  $5^{\circ}\text{C}$ ., before beginning observations. Then, with the potential divider set commonly, though by no means always, at 40 on the linear scale of 100, the current source was adjusted to a strength that just sufficed to produce anode block. With this setting it

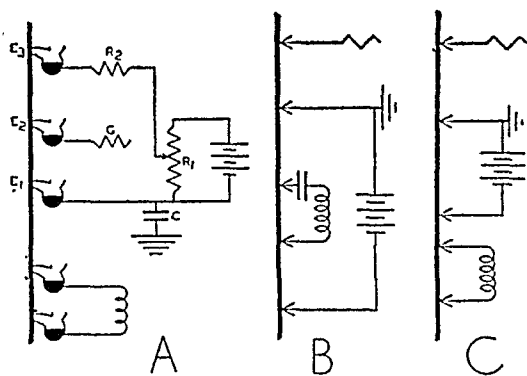


Fig. 1. NETWORKS. The resistance,  $R_2$ , was 10 megohms with single fiber preparations and 2 megohms with sciatic nerves.

usually was possible to obtain current enough to attain cathode block within the range of the potential divider.

Since these tenuous preparations tend to dry it was necessary to carry through each set of observations as quickly as possible. For this reason the successive increments or decrements of current strength were made in steps of 10th of the range subtended by the potential divider. At each strength of applied current, both anodal and cathodal, the polarizing circuit was closed, and, after the attainment of amplifier equilibrium (a matter of a few seconds) two or three records were made of the conducted spike. Additional normal spikes were recorded at frequent intervals. A complete set of observations required about a half hour in the making. Often the set of observations was repeated after rewetting the nerve and chamber. With the high amplifications needed the noise level sometimes amounted to as much as 25 per cent of the height of the lowest spikes of a series—those recorded under strong cathode polarization. To minimize error due to this and to other sources it would have been desirable to have had several readings, not only two or three, at each polarizing current strength, but, as mentioned above, expedition was necessary.

In the great majority of cases the duration of the flow of the polarizing current beyond the time required for the establishment of amplifier equilibrium, a matter of about 3 seconds, was without effect on spike height. When, as occasionally has

happened, the height of the normal spikes, due presumably to drying, has increased progressively as the experiment has proceeded, the normal heights were plotted against the approximate times they were recorded and the normal height for each of the intermediate observations was obtained by interpolation. Observations were confined to the effects produced at the polarized locus. This was necessary because the toe nerve does not have an unbranched stretch that is adequate for comparative observations at different distances from the lead, but, more important, because of the risk of bringing spikes of other fibers into the picture. Sample records are shown in figure 2.

#### EXPERIMENTAL

*Single Fiber Observations.* Within ranges to be mentioned, the data on the relation between spike height and current strength with but few exceptions describe two

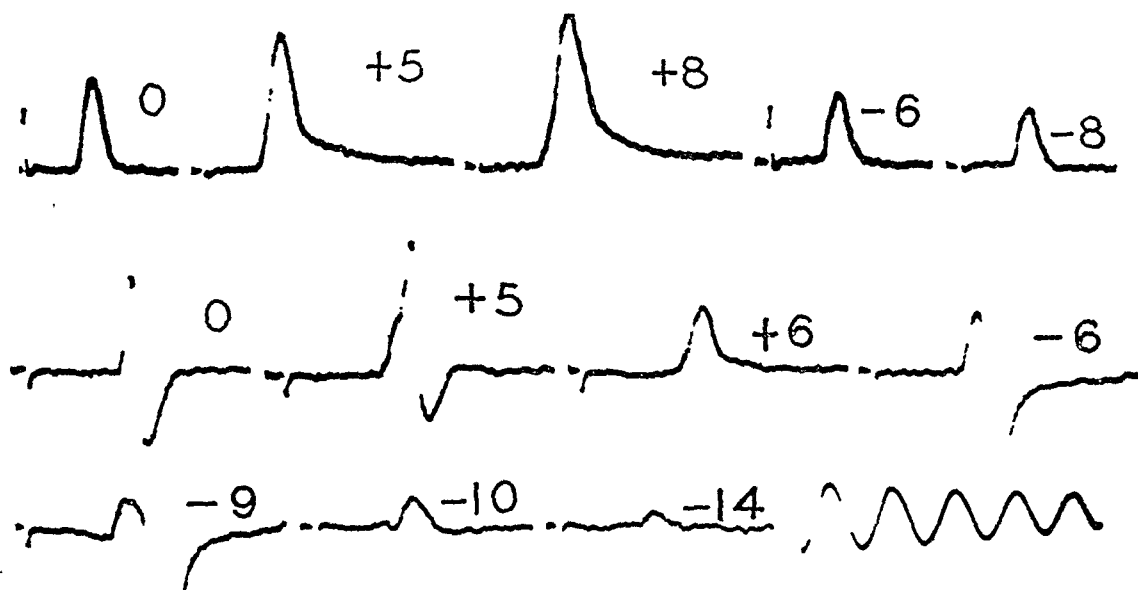


Fig. 2. A FEW OF THE SINGLE FIBER RESPONSES from two typical experiments. The numerals give the voltages in arbitrary potentiometric units. *Top row:* from experiment 6/17/48, monophasic recording. *2nd and 3rd rows:* from experiment 7/8/47, diphasic recording. 0, normal; +5, anodally polarized just short of block; +6, blocked anodally. -6, -9, -10, -14: increasing cathode polarization; -10, after block at notch near top of -9. Time, 1000 d.v./sec. Size as originally photographed.

straight lines meeting at zero at an angle that opens upwards (fig. 3 *B, C, D* and *E*); but in 3 of the 38 observations the graphs, again within the ranges to be mentioned, consisted of a continuous, inclined straight line passing through zero (fig. 3 *A*). If, within this range, there is any significant and regular departure from linearity on either side of zero it consists of a slight upward concavity on the anode side of the graphs. The eventual departure from linearity with further increase in current strength occurs, with but few exceptions, when the spike gives evidence of developing block. With anode polarization this almost invariably consists of the appearance of a notch somewhere on the up-stroke of the spike, as seen in figure 2, 2nd row, +5. At this time the rate of increase in height usually slows somewhat, but it may increase. More often, however, the linear increase continues quite to the point of block, when there occurs an abrupt and large fall in height (as from +5 to +6, fig. 2). No

attempt has been made to ascertain the exact configuration of the curve immediately preceding this event. The height attained by the spike at the time of definite deviation from linearity during anode polarization has, in this series, ranged between 118 and 182 per cent of the normal, with a mean of 151, an average deviation of 13.6 and an average deviation of the mean of 2.35. In the few cases in which the departure from linearity consisted of an upward bend of the curve the ultimate heights attained were greater even than the maximum just mentioned.

With cathode polarization the eventual departure from the linear decrease in spike height likewise may consist either of an upward (fig. 3 *D*) or a downward bend (fig. 3 *C*) of the curve. One of the factors that seems to determine the direction

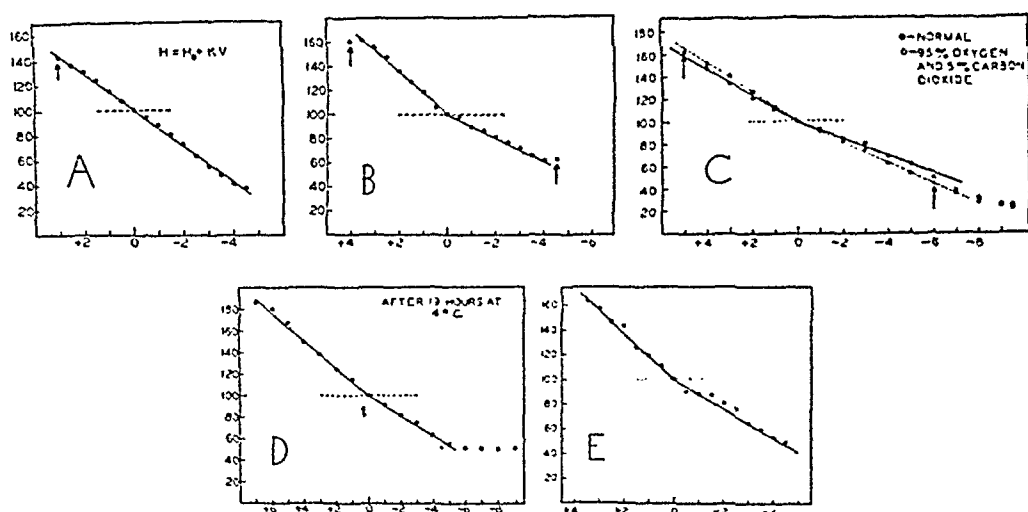


Fig. 3. GRAPHS OF SINGLE FIBER RESPONSES. *Ordinates*: spike height in percentage of normal. *Abscissae*: applied voltage in arbitrary potentiometric units. *A*, 6/12/48, normal. One of the three instances in which there was no rectification (tangent ratio of slopes = 1). Spike was notched at +3, block at +3.5; yet not blocked at -4.5. *B*, 6/17/48, normal. Spike was notched at +4, and at -4.5; not carried to block. Tangent ratio = 1.88. *C*, 5/22/48. Dots: nerve in air; tangent ratio = 1.47. Circles: in 95% O<sub>2</sub> and 5% CO<sub>2</sub>; tangent ratio = 1.28. Notched at +5, block at +6; notched at -6, block (probably) at -8. *D*, 6/10/48. After 19 hours at 4° C. Spike notched at +6, block at +7; spike deformed at -6, but not yet blocked at -9. Tangent ratio: = 1.44. *E*, 6/11/48. After 43 hr. at 5° C. Spike notched at +3.5, block at +4; not yet notched at -4.5. Considerable irregularity, but still an angle at zero. Tangent ratio = 1.59.

of the bend is the position on the spike at which the notch develops that presages the development of cathode block. If, as in -9, figure 2, the peak in front of the notch at which block will eventually occur is higher than the peak behind it, the curve of spike height is apt to bend upwards since the part of the fiber then determining the height of the spike is not so directly under the influence of the polarizing current; and if at the time of block the second peak is determining the spike height there will, of course, be a downward bend of the curve with the onset of block.

On the basis of some excitability determinations we have made it may be concluded that anode block occurs when the current attains the strength of 1 to 2 rheobases of the fiber in question, and cathode block at something less than 2 to 4 rheobases. Data supplied by 10 of the experiments show that the current strength required to produce cathode block averages 180 per cent of that producing anode block.

The current flow at the time of block is irrelevant, since it will vary with the size of the responding fiber relative to the cross-area of the nonresponding parts of the inter-electrode stretch. However, it has been of the order of tenths of microamperes.

In order to provide a means of comparing the results of the experiments in which the relevant data yield two straight lines meeting at zero strength, the ratios have been determined of the tangent of the angle formed with the horizontal by the linear portion of the curve expressing the results of anode polarization (in the 2nd quadrant) and of the angle (in the 4th quadrant) formed by the linear portion of the curve expressing the results of cathode polarization, each in relation to spike height. The data thus treated become independent of units of measurement. A ratio of one signifies that the current-strength spike-height curve is a continuous straight line passing through zero; values greater than one signify that the angle formed at zero opens upwards; while values less than one would have signified that the angle opens downwards, but of the latter there have been no instances.

Thirty-four of the 38 observations on single fibers supply data relevant to this analysis. The tangent ratios derived from one and the same preparation under constant conditions are reasonably constant, but those from different preparations have ranged between 1 and 3.64. We have been unable to ascertain definitely the factors that determine these differences. In general, however, the lower ratios are found in experiments in which the recording was 'monophasic.' Thus in the 13 of such cases, the tangent ratios ranged from 1 (and included all three of the valid cases with this ratio) up to 2.41; but this, the highest, value was obtained from a preparation, which, though crushed at the distal lead, still yielded a diphasic record; and the next highest of these ratios, namely, 2.36, was obtained from a preparation that was monophasic, but not designedly so. The average of all of the ratios derived through monophasic recording was 1.53.

There are 28 instances in which the recording was diphasic, and in most of these the interlead distances were adequate for the complete recording of the up-stroke of the spike, as shown by calculation based on the duration of the up-stroke and the conduction velocity. In these the ratios ranged from 1.17 up to 3.64, and the average was 2.24.

Attempts were made to ascertain whether prolonged storage of the toe nerves in Ringer's solution at 5° C. affects their reaction to polarization. However, in only four of many trials did the preparation survive this treatment. In three of these cases, two after refrigeration for 19 hours (fig. 3 D), the other for 43 hours (fig. 3 E), the graphs were in every respect, quantitatively as well as qualitatively, like those derived from the fresh toe nerve preparations (fig. 3 B, C). The tangent ratios were 1.44, 1.59 and 1.81. The fourth preparation, refrigerated for 4 days, gave an anomalous result: each of three complete runs yielded curves that are best described as continuously concave upwards; the spikes attained a height of 160 per cent of the normal on the anode side before block and 73 per cent on the cathode side when the current had not yet attained blocking strength. There was no angle at zero.

*Effect of CO<sub>2</sub>.* In three of the experiments observations were made first with the nerve in air and then, after the attainment of equilibrium, in an atmosphere consisting of 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub>. In two of the instances the recording was di-

phasic; in the other, monophasic. In one of these experiments the spike was deformed, making accurate analysis impossible, but there was no reason for believing that the result in this case differed in any essential manner from the consistent results of the other two experiments. The tangent ratios in the two completely satisfactory observations were, respectively, 2.19 in air, 1.95 in CO<sub>2</sub> and 1.17 in air, 1.28 in CO<sub>2</sub> (fig. 3 C). In both cases the ratios are slightly smaller in the CO<sub>2</sub> treated nerves, but the differences are small and can hardly be regarded as significant.

That the CO<sub>2</sub> was exerting its characteristic effects is indicated by two, possibly three, of the reactions. 1) In all three of the cases the shock artifacts were larger during the exposure to CO<sub>2</sub>, and in two of the cases it was noted that the stimulating

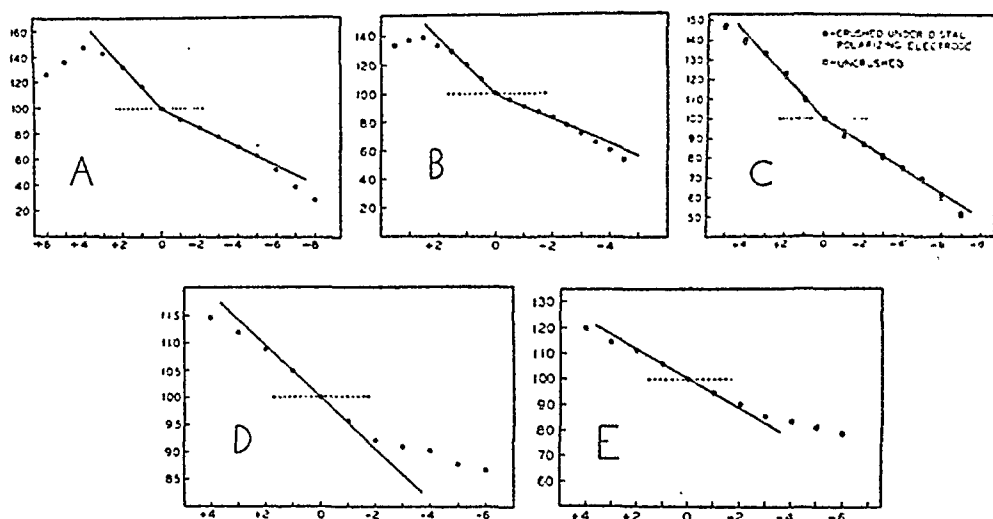


Fig. 4. GRAPHS OF SCIATIC NERVE EXPERIMENTS. *Ordinates*: spike height in percentage of normal. *Abscissae*: applied voltage in arbitrary potentiometric units. A, 5/11/49, A.M. Fresh bullfrog sciatic. Probably departing from linearity after height 118% and after height 62%. Tangent ratio of slopes = 2.14. B, 5/11/49, P.M. Bullfrog sciatic, mate of A, after storage 6½ hours in Ringer's solution at room temperature. Probably departing from linearity after height of 120% and after 78%. Tangent ratio = 2.2. C, 7/7/48. Fresh bullfrog sciatic. Probably departing from linearity after height 125% and at height 69%. Dots: after crushing under distal polarizing electrode. Circles: before crushing. The absolute voltage steps are considerably smaller in C, D and E than in A and B. Tangent ratio = 1.73. D, 7/10/48. Bullfrog sciatic after 2 days in Ringer's solution at 5° C. Inverted S in shape; no angle at O. E, 7/15/48. Bullfrog sciatic after 8 days in Ringer's solution at 5° C. Inverted S in shape; no angle at O.

current had to be increased, both observations indicating that the CO<sub>2</sub> had raised the fiber's threshold (7). 2) In all three of the experiments the CO<sub>2</sub> increased the susceptibility of the fiber to anode polarization as indicated by the strength of current required to block the impulses (2). However, the ratio of anode current to cathode current at blocking strengths in the CO<sub>2</sub> treated nerves was, as in normal nerve, approximately as 1 is to 2 (3). In all three of the experiments the curves derived from the CO<sub>2</sub> treated preparation show a slight clockwise rotation around zero (fig. 3 C). Possibly this is indicative of a change in the resistance of the nerve with a corresponding change in flow of polarizing current through it.

Alteration of other conditions, such as temperature or doubling the concentration of KCl in the Ringer's solution bathing the nerve (one experiment each), did not alter appreciably the form of the graphs or the tangent ratios.

*Observations on Sciatic Nerve.* For purposes of comparison, similar data have been obtained from the unbranched stretch of the sciatic nerve of the frog. The stimuli were 10 times half maximal for alpha. Since the primary aim was to ascertain whether graphs based on data derived from multifibered preparations also are linear on either side of 0, and, if so, through what range, the current strength, as a rule, was carried through a rather narrow range,—only twice to the point where there was an actual decrease in the height of the spike with anode polarization (fig. 4 A, B).

*Relatively freshly prepared nerves* were used in 10 of these experiments, 9 from the bullfrog and 1 from the green frog. Observations were begun usually within an hour after the dissection. In seven of the experiments the recording was diphasic, and the interlead distances (21 to 35 mm. in the bullfrog) were ample for the recording of the complete up-stroke of the spike. In the three experiments with monophasic recording the interlead distances were 10 mm. in two of the cases and 12 mm. in the other.

In the graphs of the data derived from the 7 preparations with diphasic recording and from the monophasic preparation with the 12 mm. spacing of the leads, the middle sections again consist of two straight lines emanating from zero at an angle (fig. 4 A, B, C). The tangent ratios of the slopes were 1.54, 1.70, 1.73, 1.76, 1.85, 2.14, 2.22 and 1.82 (the last from the green frog); there still is some variation in the width of the angle from preparation to preparation but the range is narrow in comparison with that obtaining in the case of the observations on single fibers. The two nerves yielding the tangent ratios of 2.14 and 2.22 were from the same bullfrog; the first (fig. 4 A) was used shortly after preparation, the second (fig. 4 B) after storage for 6½ hours in Ringer's solution at room temperature. Excepting a slight counter-clockwise rotation of the graph derived from the stored nerve, which could be due to a difference in the potential subtended by the potential divider, the two graphs and the two tangent ratios are essentially alike.

In those instances in which the strength of the polarizing current was carried to the point where there was a change in the rate of change in height, the initial departure from the linear, as with single fibers, consisted sometimes of a slight upward bend of the curve, sometimes of a downward bend. With diphasic recording and anode polarization this bending of the curve occurred at approximate spike heights of 112, 114, 118, 120, 127 and 133 per cent of the normal. These values, obtaining at the time of changing inclination of curves, are roughly of the same order of magnitude as the lowest values found in the comparable observations on the responses of single fibers. In the two instances in which anode polarization was carried far enough to cause a marked decrease in spike height (fig. 4 A, B) this decrease began when the heights attained were 140 and 147 per cent of the normal. With cathode polarization the bending of the curve developed when the heights attained were approximately 62, 69, 74, 78, 82 and 84 per cent of the normal.

The curves derived through monophasic recording were not consistently alike. When the interlead distance was 12 mm. the curve mounted straight to a height of 148 per cent, and under cathode polarization it departed from linearity at a height of 64. However, the curves derived from the two experiments with the interlead distance of 10 mm. differ completely from the form that is usual, one of the graphs consisting of two inclined parallel, nearly straight, lines, the continuation of the 'anode' line lying

slightly above the continuation of the 'cathode' line, the other consisting of a curve slightly and continuously concave upwards.

Similar observations have been made on *bullfrog sciatic nerves after storage in Ringer's solution* at about 5° C. for periods ranging from 1 to 8 days. (A 12-day nerve was inexcitable, and, as mentioned above, a 6½-hour nerve behaved quite like its fresh mate.) In 8 of the 9 experiments the recording was diphasic with interlead distances ranging between 24 and 34 mm. Within the relatively narrow range of current strengths used in this series of experiments, the graphs derived through diphasic recording have, or approximate, the form of the middle section of an asymmetrical, reversed S: the curves (fig. 4 *D, E*) pass through zero with a gradually changing slope, the ordinates on the anode side usually increasing somewhat faster with increasing current strength than on the cathode side. The change in slope on either side of zero is so gradual that the middle sections of the curves can be regarded as essentially straight. In the one case with monophasic recording (a 3-day nerve with interlead distance of 8 mm.) the graph consisted of an inclined straight line passing through zero and bending upwards at both ends. Conduction velocities were of the same order of magnitude in the stored as in the fresh nerves.

#### DISCUSSION

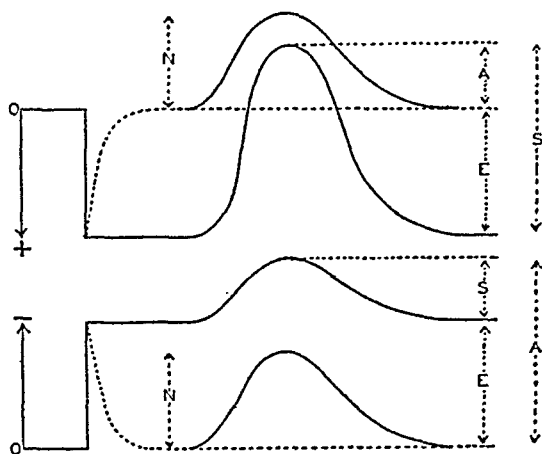
The first of our observations that requires consideration is the finding that the height of the conducted spike of a single fiber as recorded at the polarized locus, within certain limits, varies linearly with the strength of the polarizing current on either side of zero. In this connection it is necessary to recall how a resistance-capacity coupled amplifier performs under our experimental conditions. It records the spike but not the electrotonic potential that is established some 3 or more seconds earlier, since, in the interval, the condensers have filled and the base line consequently has returned to zero, where it is when the spike records. At the same time, however, the membrane potential, with certain qualifications (2), has been changed by the algebraic addition to it of the electrotonic potential; and when the membrane breaks down the height of the spike that results is the sum of the electrotonic potential and the potential of alteration or action, as illustrated in figure 5.

Since, in our experience, the relation between the height of the spike of a single fiber and the applied current, both positive and negative, is within certain limits linear and since, in our experience, the same relation usually obtains also in the case of the fresh sciatic nerve of the frog, though usually through narrower limits, it is logical to conclude that in these preparations *all* of the processes that are altered by the applied currents are altered linearly within those limits. This conclusion is inconsistent with the relevant results Lorente (2) has obtained in his polarization experiments on the sciatic nerve of the frog as seen in the two of his graphs (vol. II, figs. 1 and 2, pp. 36 and 37) illustrating the relation between the electrotonic potential (not spike height) and the strength of an applied current. Of these his figure 1 shows this relation as determined at the polarized locus with the multifibered preparation in air, and his figure 2 the result as obtained at a distance of 3.5 mm. from the polarizing electrode with the nerve in an atmosphere consisting of 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub>. When these two curves of Lorente are plotted as we have plotted our data,

namely, the results of anode polarization in quadrant 2 and those of cathode polarization in quadrant 4, it becomes evident that the resulting curves have the form of a reversed S, are without an angle at zero, and consequently closely resemble in form the middle sections of the curves of spike height against polarizing current that we have obtained only, and practically without exception, from the bullfrog's sciatic nerve after storage in Ringer's solution at 5° C. for 24 hours or longer. The latter result, it might be added, serves as a check on our methods, since it shows that they are capable of exposing a non-linear reaction to polarization when it exists.

Lorente's graphs indicate that the deviation from linearity of the relation between strength of polarizing current and electrotonic potential is particularly marked in nerves exposed to an atmosphere containing CO<sub>2</sub>. It was for this reason that we performed, with the single fiber preparation, the comparable experiment—the determination of the effect of CO<sub>2</sub> on the spike-height current-strength relationship. The addition of the CO<sub>2</sub> did not demonstrably alter the linear relationship from

Fig. 5. DIAGRAM ILLUSTRATING MODIFICATION of height of a spike as recorded by a condenser coupled amplifier when equilibrium has been established after the addition of a constant source of potential. *S*: recorded spike height. *A*: potential of alteration or action. *E*: electrotonic potential. Spike height during anode polarization =  $A + (+E)$  and during cathode polarization =  $A + (-E)$ .



that found with the nerves in air. This observation should not be taken to signify that CO<sub>2</sub> never alters this relationship, since it is possible that with us the nerves prior to their exposure to CO<sub>2</sub> may have been in a state similar to that produced by CO<sub>2</sub>. That, however, the treatment with CO<sub>2</sub> did change the state of the fibers characteristically is proved by an associated decrease in their excitability, by an increase in their relative susceptibility to anode polarization and by a clockwise rotation of the graphs around zero.

As has been said, with single-fiber preparations this linear relation on either side of zero holds up to current strength that block the nerve impulse, or that begin to produce the deformations of the axon spike that presage block. Recently Rosenblueth *et al.* (8) have questioned the evidence upon which the conclusion was reached in this laboratory (3, 6) that the one or two successive 'quantal' changes in the spike of a single fiber that occur as the strength of a polarizing current is increased are due to block. They say that when, in their experience, they were certain that only one fiber was responding, in no case did they see sudden decreases in the spike, even when they polarized anodally or cathodally until total extinction of the response took place. Ignoring the conclusive portion of our published evidence proving that the 'quantal'

changes we have described are due to block in a single fiber, they assert that the pictures we have published as evidence of that are the result, not of block in one fiber, but of the play of the responses of two fibers.

As proof of this assertion they present two records (their fig. 1 *C, D*), both from the same root preparation, which they say "are quite similar to" the pictures which we have accepted as evidence of block in a fiber. We can agree with them that *C* and *D* are made up of the responses of two fibers; no one could possibly have regarded them as responses of but a single fiber. That *C* is made up of two fiber spikes is proved, as they point out, by the presence of tell-tale latency shifts of only the upper part of the record, indicating that the stimulus is just threshold for the fiber making that contribution to the picture, but well above the threshold of the fiber making the lower contribution. That *C* and *D* each is composed of the spikes of two fibers is indicated also by the position of the notch on the up-stroke—it is too far in front of the crest of the lower spike. In polarization block of a single fiber the part of the picture about to disappear rises, typically, out of the crest of what remains at block. Even in experiments in which the conduction distance is as long as 130 mm., as was the case, approximately, in all of our earlier experiments, the divergence from this relation was rarely greater than 0.2 msec. (3). The conduction distance in the case of their figures *C* and *D* could not, with their preparations, have been greater than 35 mm.; yet the upper spike of their figure rises out of the lower 0.25 msec. ahead of the crest.

In our experience, the really difficult double-fiber response to identify as such is when there are two fibers with identical excitabilities and conduction velocities. But even under such circumstances, it must be rare, indeed, that during the course of a prolonged experiment, involving the appearance on the screen of many hundred threshold responses, something does not transpire to disclose the compound nature of the response. Their "good test for the all-or-nothing character of the response" differs from the one we have used through the years and in the present research, only in respect to the frequency of stimulation; their threshold stimuli are at the rate of 50/sec., ours about once per sec., but through long periods of time.

Additional evidence (all of which is on record) on which we base the conclusion that the 'quantal' changes in the spike are the result of successive blocks along the course of a single fiber is, to consider now for the sake of brevity only the effects of anode polarization, as follows:

1) It is not unusual for the 'quantal' changes, when there are two of them, to reduce the height of the spike to  $\frac{1}{6}$  or even to  $\frac{1}{8}$  its initial value (3), *and this residue, moreover, is anodally polarized*. If such 'quantal' decreases were the expression of the elimination in succession of the spikes of different fibers, then, since there is in general a direct relation between spike height and conduction velocity (5), the conduction time should increase markedly with each defection, but if it changes at all the change is in the direction of a slight decrease.

2) Needless to say, if the two or three sudden changes in the spike that occur as polarization is increased were pictures derived from as many fibers, the fibers would have very different diameters and consequently correspondingly different thresholds, but the threshold remains the same throughout such an experiment.

3) The information supplied by records obtained with diphasic recording is unmistakable, yet Rosenblueth *et al.* fail to mention this phase of our published evidence. When a single fiber is responding the invariable result, provided the increase in current strength is carried far enough, is that with the first 'quantal' change the record becomes completely monophasic—the impulse is completely blocked (cf. 'S<sub>2</sub>' spikes of fig. 1, 6). If, with further increase in current strength a second and a third abrupt decrease in height occurs, the quantitative relationships are the same as with monophasic recording, and what we have said in that connection applies here. Now if, say, two sudden 'quantal' decreases in spike height were the expression of the participation of 3 fibers in the initial pictures, one would expect the largest fiber to have not only the fastest conduction velocity but also the greatest susceptibility to polarization (9). On this basis it should be the first to block as the current strength is increased, and at that stage the spikes of the still unblocked smaller fibers should remain diphasic and become monophasic only after further increases in the strength of the polarizing current; but, as a matter of fact, the change to monophasicity invariably is all or none when the response is that of a single fiber. There are, however, conditions under which a blocked impulse can re-initiate the impulse beyond the block (6); those conditions are of no present concern.

Rosenblueth *et al.* (8) say that as a means of securing the responses of a single fiber their spinal root preparation is a 'compromise' between giant fibers and our toe nerve preparations. This, they imply, is because their preparations are less than 70  $\mu$  in diameter and were found to contain 'several' functional nerve fibers. The toe nerve preparation at the point led from has a diameter of 20 to 50  $\mu$  and by actual count contains 20 to 100 fibers ranging down from a diameter of 12  $\mu$  (5). A root preparation 70  $\mu$  in diameter could contain more than 27 fibers measuring 10  $\mu$  in diameter. Under the circumstances, can it be assumed that the word 'several' implies that only a few of the constituent fibers of root preparations conducted from end to end? If so there can be but little difference between their root preparation and our toe nerve and split sciatic preparations with respect to *a*) the number of contained fibers or *b*) the possibility of local variations in diameter and associated local demarcation currents which might facilitate the development of local blocks under polarization.

Rosenblueth *et al.* argue, as does Lorente also (2), that the myelin sheath could not act as an insulator. In support of this position the former refer to the experiments of Tasaki (10) indicating that myelin is not a perfect dielectric, but not to the statement by Tasaki in the same paper that "Die internodale Strecke der Faser lässt sich nicht erregen," nor to a later publication by Tasaki *et al.* (11) in which it is stated that the action current of a nerve fiber derives from the Ranvier nodes and not from the myelin-covered region of the fiber. Moreover, complete support for our conclusion with regard to the location of blocking points at nodes is supplied by the experiments of Huxley and Stämpfli (12) who find that the membrane current through the myelin can be explained as a passive current through a resistance and capacity in parallel, and conclude "that the action potential at each node excites the next node by currents flowing forward in the axis cylinder and back in the fluid outside the myelin sheath." This result in some respects is similar in its implications to results

obtained by Blair (13). The latter found that "in depressed nonconducting medullated nerve, the all-or-nothing response of a single segment is the least obtainable; increasing the strength of stimulation increases the spike through the entrance of additional segment responses."<sup>3</sup>

Huxley and Stämpfli found that immersion of a nerve fiber in oil alters its reaction to isotonic sugar solutions; so, suspecting that the oil might affect also the distribution of current lines, they devised a method that made it unnecessary to bring oil into contact with the fiber. In attempting to account for the differences between Rosenblueth's and our results a similar thought had occurred to us, also. In any event, however, the behavioral differences of these two preparations, the phalangeal nerve and the spinal root, under polarization remain to be reconciled.

Within specified limits, the graphs derived from single fibers and from fresh, multifibered preparations are described, with but few exceptions, by the formula  $h = h_0 \pm kv$ , where  $h_0$  is the height of the normal spike,  $v$  the polarizing voltage, the constant  $k$ , which certainly must be a function of the membrane resistance, being as a rule larger, but never smaller, when the polarizing electrode is the anode. The latter result is in keeping with the long known fact that the electrotonus produced by anode polarization is greater than that produced by cathode polarization, a difference that is usually regarded as evidence of the action of the membrane as a physical rectifier, but, by Lorente, as a reaction on the part of nerve.

To refer to some of the more recent of the relevant literature dealing with this subject, Cole and Hodgkin (14), using single giant nerve fibers of the squid and alternating current bridge methods, at first were unable to demonstrate 'rectification.' Then Cole (15) found that it could be demonstrated by killing the nerve under one of the polarizing electrodes and concluded that in their first set of observations the rectifying action had been concealed by approximately equal and opposite rectifications at the anode and at the cathode. Another reason, Cole believed, was failure in their earlier experiments to use polarizing currents of adequate strengths. Then Guttman and Cole (16) found that after killing the fiber at one of the polarizing electrodes the 'rectification' effect could be directly observed through the use of a direct current Wheatstone bridge. In the present investigation killing the locus of the toe nerve or of the fresh sciatic nerve over the distal polarizing electrode (with arrangement A, fig. 1, and diphasic recording) has not altered consistently the width of the angle at zero, although, owing to the proximity of the polarizing electrode to the distal lead, the diphasicity often was reduced somewhat thereby in the case of the toe nerve. In this connection it may be mentioned that according to Hodgkin (17) the membrane of the giant fiber of *Carcinus* obeys Ohm's law over a wide range of anodic current but shows marked deviations with cathodic currents. In the latter respect his result differs from that found here with frog's nerve.

The wide range, in different single fiber preparations, of the width of the angle formed at zero in the graphs of the relation between current strength and height of spike probably is due, at least in part, to variations in the extent of injury inflicted during the making and handling of the delicate preparations. As has been mentioned, this is indicated by the fact that statistically the ratios of the tangent of the

<sup>3</sup> Additional references relative to this subject are given by Huxley and Stämpfli.

angles formed with the base line of the anode and of the cathode lines are definitely smaller, on the average, with monophasic than with diphasic recording. Damage could have this effect if it diminished the rectifying action of the membrane. The much greater uniformity of tangent ratios derived from observations on the sturdier fresh sciatic nerve is in keeping with this view. It is a matter of some interest, however, that nerve fibers appear to be functioning quite normally through a range of rectification, such as is represented by tangent ratios of 1, or no rectification, and 3.6.

Stray demarcation currents, such as might result from local damage or from cut branches, cannot be a factor in this connection since any such would add themselves algebraically to the applied currents and the only result would be to shift the graphs laterally, so that the angle would no longer be at the zero of the applied current. Only one probable instance of this has been seen. That stray demarcation currents are not a main factor, if they are a factor at all, in the production of the angle is demonstrated by the presence of the angle in the experiments with fresh frog sciatic nerves. Indeed, it is worthy of note that in diphasic recordings the averages of the tangent ratios derived from multifibered (1.84) and single fiber (2.20) preparations are essentially alike. This is all the more remarkable in view of the fact that the multifibered spike is the average of the spikes from a wide range of fibers whereas the single fiber spike is probably that of a fiber larger in diameter than the average.

Tasaki *et al.* (18) have recently investigated the modification of the electric response of a single node of Ranvier by polarization. They, as we, used a resistance-capacity coupled amplifier. According to them, cathode polarization decreases spike height markedly, while anode polarization increases it, but only slightly. These results, they point out, and correctly, differ from those of all other investigators, and they ascribe the "discrepancies . . . to the complex character of the electrical network in the nerve trunk, especially to the highly polarizable character of the myelin sheath of the fiber in question or of the neighboring fibers". It seems more likely that their results differ from ours because of differences in networks and also because of differences in the time and the place of application of the currents employed. In their experiments the cathode of the stimulating circuit and the proximal recording and polarizing electrodes were one. The polarizing current was started only 11 msec. "before the onset of the long rectangular current pulse by which the spike to be recorded, was evoked." The duration of their stimulating current is not stated in this particular connection, but earlier in the paper they speak of "a rectangular current of 10 msec. duration." Under such conditions the stimulating current would add to the effect of the polarizing current when the latter was cathodal, and subtract from the effect of the polarizing current when it was anodal. It is just in these respects that the results pictured in their graph (their fig. 2 C) differ from ours: on the anode side of their graph the slope is slight (the increase in spike height is slight), whereas on the cathode side there is a steep decline. Their graph on the anode side seems to be concave upwards, but not clearly so because of the narrowness of range of amplitudes. On the cathode side their curve opens downwards, but the scattering of points is quite wide; consequently it is almost possible to regard their total graph as being composed of two straight lines meeting with an angle at zero, but with a *downward* bend there.

As to differences in the time relations of current application, their polarizing current, as stated above, starts only 11 msec. before the onset of the rectangular stimulating current through the common electrode. In our experiments the action potential is conducted to the polarized locus and arrives there several seconds (not msec.) after the start of polarization. But whether these temporal differences also are a factor in the production of the results they get, it is impossible to say.

The plotted data of our study yield curves of three types: *a*) There is the single-fiber type, linear on either side of zero up to polarizing current strengths that begin to deform the spike, or that actually block the impulse; this occurs at spike heights that range widely, between 118 and 182 per cent of the normal under anode polarization and between 19 and 60 per cent under cathode polarization. This type is not altered by storage of the preparation in Ringer's solution. Then there are multi-fibered types of two kinds. *b*) With fresh preparations the result again is linearity on either side of zero, but through a narrower range of change in spike height, the ultimate gradual departure from linearity occurring at heights of 113 to 133 per cent with anode polarization and 74 to 84 per cent with cathode polarization; i.e., at levels corresponding roughly with the lower levels of departure from linearity in the case of single fibers. *c*) With multifibered preparations stored 24 hours or longer the middle portions of the curves approach the shape of a reversed S, passing through zero almost linearly, i.e., without a sharp change in direction there, simulating in shape the curves of electrotonic potential published by Lorente.

Curves of type *a* could signify that the action of the current on a normal fiber is exerted primarily at a specific, the most accessible, locus until block supervenes there, that then the action of the current is displaced to the next, a more remote, most accessible locus, and so on. These loci might be either nodes of Ranvier or entire internodal segments. Curves of type *b* then would be the resultant of a multitude of curves of type *a*. And the disappearance of loci of higher susceptibility to current action, such that all parts of each fiber become equally susceptible, would result in curves of type *c*.

Possibly relevant to this view is the observation of Weddell and Glees (19) that in warm-blooded animals section of a nerve *in situ* results in structural changes in the myelin sheath that become obvious within 12 hours. Such changes might render the axon more accessible to applied currents and equally so everywhere. If this occurred within 24 hours in the case of the stored sciatic nerve of the bullfrog, curves of type *c* would be accounted for, since under these conditions spike height would change logarithmically with changes in the strength of the polarizing current. A much longer period of storage would be required to produce these changes in the case of the toe nerve because, due to its tenuousness, there would be less interference with its metabolism under these conditions.

There have been occasional variations from the rules as stated above. For these we have no explanation to offer. Our experience in this respect is not unique. Lorente, for example, notes that exposure to a given concentration of CO<sub>2</sub> may yield values in different nerves that differ by as much as 50 per cent under apparently like conditions (2).

## SUMMARY

Determinations have been made of the effect of polarization on the height of the spike at the polarized locus, after amplification with a resistance-capacity coupled amplifier, of single medullated fibers of the phalangeal nerve and of preparations split from the sciatic; and, for purposes of comparison, similar observations have been made on multifibered responses from the unbranched part of the sciatic nerve of the frog, both freshly dissected and after storage in Ringer's solution at 5° C.

The height of the single fiber spike, with very few exceptions, increases linearly under anode, and decreases linearly under cathode polarization up to current strengths that produce signs of, or actual, block. The proportionality factor rather regularly is greater (but never smaller) for inwardly directed currents. The ratios of these factors range rather widely from preparation to preparation, due, possibly in part, to varying amounts of local damage done during preparation, though no sign of altered functioning is apparent even when this evidence of rectification is absent. The height of the spike in different preparations at the limits of linearity, in percentage of the normal, has ranged between 118 and 182 with anode, and between 19 and 60 with cathode, polarization. Storage of this preparation at 5° C. up to 43 hours does not alter the result appreciably. The evidence that we are dealing with the responses of single fibers is reviewed.

With the freshly prepared sciatic nerve the relation on either side of zero polarization likewise is linear in a majority of the cases. In such cases the range of rectification, as measured by the tangent ratios of the slopes to either side of zero, is relatively narrow, but the departures from linearity of the relation of spike height to polarization strength begin at relative spike heights that are roughly the same as the lowest of the values derived from single fibers.

Sciatic nerves stored 1 to 8 days regularly yield curves of the current strength vs. spike height which in their mid sections are similar in configuration to published curves of the relation of electrotonic potential to strength of polarizing current; they are reversed S in shape, pass through zero polarization without an angle, but with ordinates that are somewhat larger with ingoing than with outgoing currents.

These results are consistent with the view that in a normal, segmented, peripheral nerve fiber all of the susceptible processes that are concerned with the determination of the height of the spike are altered linearly by applied currents up to strengths that block, or begin to block, the nerve impulse, and that this relation breaks down in nerves stored under relatively anaerobic conditions, due possibly to a change in the dielectric properties of the myelin sheath.

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# EFFECTS OF CHROMATOLYSIS ON INTERACTION OF SPINAL MOTONEURONS<sup>1</sup>

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**R**ENSHAW (1) has shown that antidromic volleys condition the synaptically excited discharges of other spinal motoneurons. The most striking effect is a pronounced inhibition when the synaptically excited cells and the motoneurons receiving the antidromic volley supply the same muscle or muscle group. Thus, conditioning effects are most apparent when the two groups of motoneurons occupy the same portion of the ventral horn, axially as well as cross-sectionally. The mechanisms involved in this conditioning have been reduced to the two most likely possibilities. These include the external action potentials of the antidromically fired cells and the impulses discharged by the recurrent collaterals transmitting the antidromic volley. Axonal chromatolysis of spinal motoneurons selectively reduces the external action potential without affecting conduction along the axons or, presumably, their collaterals (2). In this experiment, the chromatolysis of the conditioning motoneurons was used to differentiate between the two conditioning mechanisms that were postulated.

In Renshaw's experiment, a proprioceptive or monosynaptic reflex discharge was used as a test volley in conjunction with an antidromic conditioning volley. A significant feature of this conditioning was the early onset of inhibition of the test response. In fact, conditioning occurred when the testing impulses, which fired the tested motoneurons after a single synaptic delay, and the antidromic volley arrived in the ventral horn simultaneously. Therefore, indirect inhibitory mechanisms were eliminated as a source of conditioning because they require a longer conditioning interval than was present in that experiment. Renshaw (1) and Lloyd (3) described the experimental conditions that were most favorable for the production of maximum inhibition. These included the use of a maximal conditioning shock and a threshold proprioceptive test discharge. A fixed antidromic volley inhibited the greatest percentage of the total neurons fired when the test shock was near threshold. As the strength of the test discharge increased beyond this point, a fixed antidromic volley inhibited an increasingly smaller percentage of the total neurons fired. On the other hand, a constant threshold test response showed a progressive decrease in amplitude as the strength of the conditioning discharge was increased. But at the point of maximum conditioning, an increase of twenty-fold in the magnitude of the conditioning shock did not increase the response deficit.

The selective functional alterations produced by axonal chromatolysis were studied by two groups of investigators. Acheson, Lee and Morrison (4) found a progressive decrease in the amplitude of the spontaneous discharge recorded from the central end of a previously cut phrenic nerve, as compared to the uncut nerve on the opposite side. This effect became apparent 8 days after section, and increased in intensity up to 21 days. In critical animals, both phrenic nerves were excised, after definitive experimentation, and studied in a moist chamber. When correction was made for con-

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duction velocity, there was no significant difference in the amplitude of the maximal spike for A fibers in the two nerves. It was concluded that the deficiency in the spontaneous firing of the previously sectioned phrenic nerve was not due to failure of conduction in the phrenic axons.

Campbell (5, 6) correlated the physiological alterations of sciatic nerve and ventral root section with the resultant chromatolysis of the nerve cells in the lumbosacral cord and spinal ganglia of cats and monkeys. He found a loss of the proprioceptive component of the segmental spinal reflex during the period of chromatolysis. This did not stem from degeneration in the sensory system, for there was no alteration in the amplitude or rate of conduction from the peripheral to the central fibers of the dorsal root ganglion cells. Furthermore, the negative cellular response of the antidromic cord potential was decreased or absent, whereas the positive axonal potential of the approaching impulse was unchanged (2). Conduction along afferent pathways and axons of involved spinal motoneurons thus was not affected in chromatolysis. In further studies, Campbell, Gasteiger and Mark (7) quantitated the effect of chromatolysis in the multi-synaptic tibial-peroneal reflex. They found a decrease in the total electrical excitability of the reflex beginning 3 days after peroneal nerve section. The time course of this altered response in the early stages of chromatolysis was paralleled by histological studies of the involved spinal motoneurons. It was concluded that the chief functional alteration found at the spinal level was an increase in the threshold of excitability of the chromatolyzed motor nerve cells.

#### MATERIALS AND METHODS

Six cats had the nerves to their right semitendinosus and semimembranosus ligated and severed with sterile precautions and under light sodium pentobarbital anesthesia, 5 to 9 days before the experiment. The terminal procedure was carried out under very light dial anesthesia which was augmented by midthoracic spinal cord transection. Dorsal roots from L-5 to S-4 were severed intradurally and bilaterally. Reflex proprioceptive discharges were produced in the nerves to the biceps femoris by stimulation of appropriate dorsal roots. Antidromic discharge was initiated by stimulation of the nerves to the semimembranosus and semitendinosus. Recordings of the antidromic cord potential were made from the mid-dorsum of the cord with a monopolar ball electrode. The usual differential amplifier, sweep synchronized thyatron stimulator and cathode ray oscillograph were used. Histological sections were taken from the lumbosacral cord.

#### RESULTS

Both histological and physiological evidence of chromatolysis were present in the motoneurons of the nerves to the right semitendinosus and semimembranosus. Tigrol-ysis of motoneurons on the right was present at the S-1 level of a 9-day animal. The location of the chromatolysed motoneurons, in cross-section, correspond to similar studies of the hamstring nuclei in dogs by Marinesco (8). The degree of chromatolysis resembled stage III of Campbell and Novick (9), with widespread dissolution of the Nissl substance and eccentricity of the nuclei. Reflex segmental discharges into the nerves of the right semitendinosus and semimembranosus were characterized by a delayed response without a discharge at the usual time of the proprioceptive deflection. In addition, the negative cellular component of the antidromic cord potential was deficient on the right. This is demonstrated in figure 1a, contrasting the right and left antidromic cord potentials in a 5-day animal with the same recording conditions.

The conditioning of a threshold proprioceptive reflex in the nerves to the biceps by a maximal antidromic volley initiated in the central end of the previously cut

nerve of the semitendinosus and semimembranosus was a pronounced inhibition in every case. In table 1 the maximum inhibitory effect is listed in 6 chromatolysis ex-

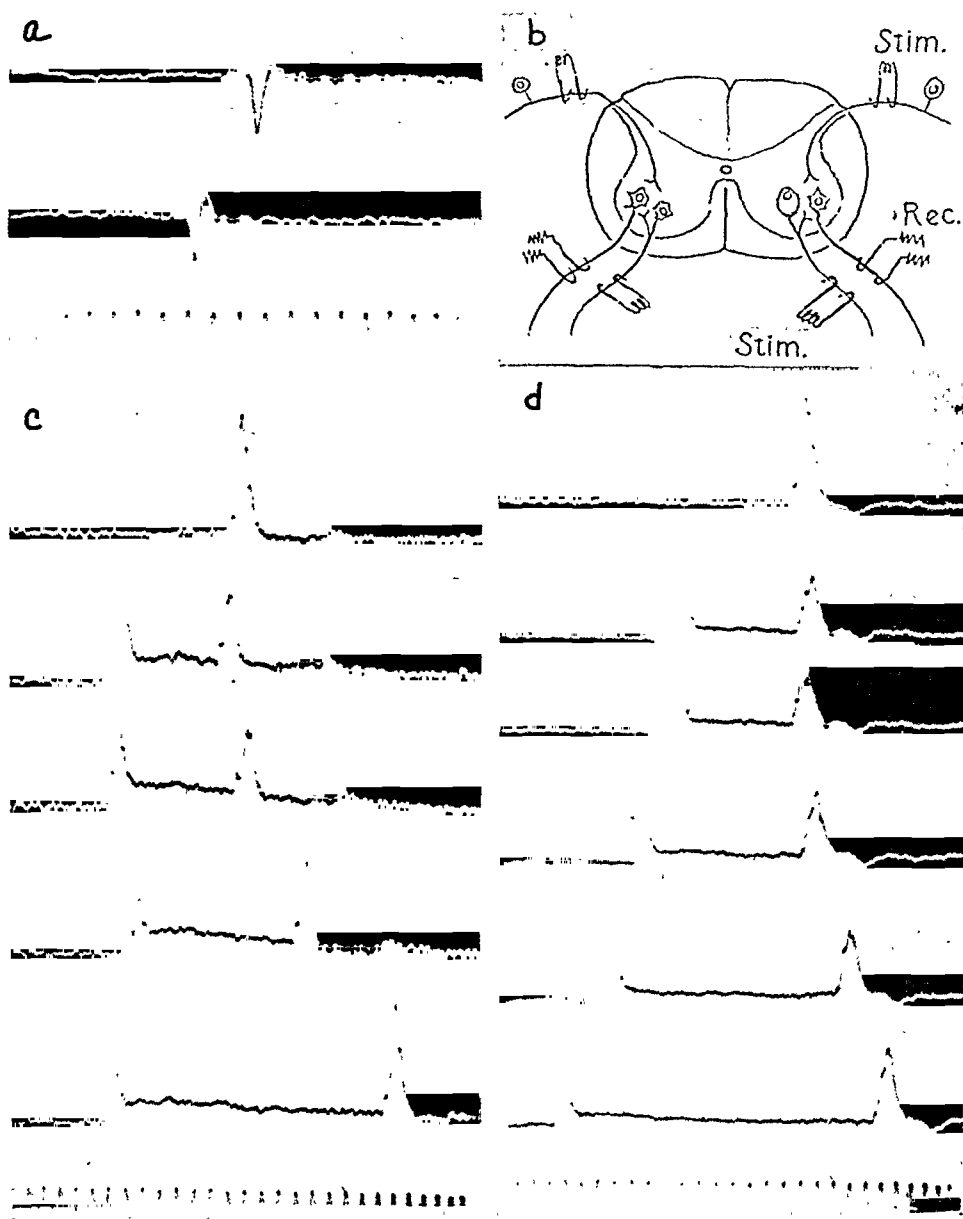


Fig. 1. *a*. COMPARISON OF ANTIDROMIC CORD POTENTIALS of chromatolysed (upper) and normal (lower) evoked by stimulation of the nerves to the semimembranous and semitendinous muscles. *b*. Scema of conditioning experiments. *c*. Conditioning effect of antidromic stimulation of normal neighboring neurons upon reflex discharge in the nerve to biceps femoris. *d*. As in *c* but with antidromic shock to chromatolyzed neurons. Time in milliseconds.

periments and compared to 5 normal experiments from this series. The maximum inhibition produced in the 6 experiments with chromatolyzed conditioning motoneurons varied from 25 to 52 per cent of the test response. The maximum inhibition in 5 control experiments varied from 25 to 56 per cent of the test response. The similarity in maximum conditioning effects is further illustrated by the oscillographic

records of the critical conditioning intervals on the control and chromatolyzed sides of a 5-day animal (fig. 1 *c, d*). These records also indicate a similar time course of inhibition on the two sides. This fact is borne out by the conditioning curves of 4 chromatolysis experiments contrasted with 2 normal conditioning curves in figure 2. Each significant point in each curve was the resultant average of between 10 and 24 alternate determinations of the conditioned and test response. The curves demonstrate the maximum biological variation obtained in the control and chromatolysis experiments. In both the chromatolyzed and the normal conditioning curves, a response deficit was present at an interval of 2 msec. These curves resemble the conditioning curves of similar experiments using the nerves to the 2 heads of the gastrocnemius by Renshaw (1) and Lloyd (3). In summary, the table of maximum inhibitions and the conditioning curves show a marked similarity in the chromatolysis and control experiments. This indicates that chromatolysis and the subsequent reduction of the external action potential of the conditioning motoneurons does not affect the magnitude or the time course of this conditioning.

TABLE 1. MAXIMUM INHIBITION LISTED AS PERCENTAGE OF TEST SHOCK

DAYS OF DEGENERATION	CHROMATOLYSIS EXPERIMENTS %	CONTROL EX- PERIMENTS %	DAYS OF DEGENERATION	CHROMATOLYSIS EXPERIMENTS %	CONTROL EXPERIMENTS %
5	38 <sup>1</sup>	52 <sup>1</sup>	9	25 <sup>1</sup>	
9	41	25	8	52	
5	52	54			58 <sup>1</sup>
8	45				30 <sup>1</sup>

<sup>1</sup> Maximum conditioning recorded only.

#### DISCUSSION

The recurrent collaterals of axons of mammalian spinal motoneurons were first mentioned by Golgi in 1883 (10). Their finding was substantiated by Ramon y Cajal (11), v. Koelliker (12) and others. According to v. Lenhossek (13), the majority of the collaterals originated from the axons as they passed from the gray to the white matter. The recurrent course of the collaterals was described by Ramon y Cajal (14). He followed these fibers to their apparent termination in the ventral horn in the vicinity of neighboring motoneurons. He was not able to give an exact description, however, of the termination of the collaterals on cell bodies or dendrites.

The functional significance of the recurrent collaterals of spinal motoneurons was discussed by v. Lenhossek in 1895 (13), who thought that they might influence the excitability of neighboring nervous elements. T. Graham Brown (15) tried to incorporate the collaterals into his theory of antagonistic half centers, as mediators of inhibitory impulses. A. Forbes (16) postulated a similar rôle for the recurrent collaterals in the reciprocal innervation of antagonistic muscles. Later, this theory was put to a careful experimental test (17). It was found that a crossed-extensor reflex, set up by contralateral sciatic nerve stimulation, was not modified by antidromic volleys arriving at the cord in motoraxons of the ipsilateral peroneal nerve. Renshaw (1) was the first to demonstrate the direct inhibitory effect of an antidromic volley on the reflex discharge of neighboring motoneurons, and he considered this effect a

mechanism for synchronization of the firing of motoneurons in a particular nuclear group.

Grundfest (18, 19) and Renshaw (1) have reviewed the biological effects of intrinsically generated action potentials on neighboring or contiguous nervous elements. The reduction of the external action potential in the present experiment, however, had no effect on the conditioning observed. Thus, in Renshaw's experiment, the possibility that conditioning effects were caused by the external action potential of the antidromically activated motoneurons is eliminated. The other inhibitory mechanism that was postulated, namely, the impulses discharged by the recurrent collaterals, could not be subjected to selective experimental evaluation in the spinal

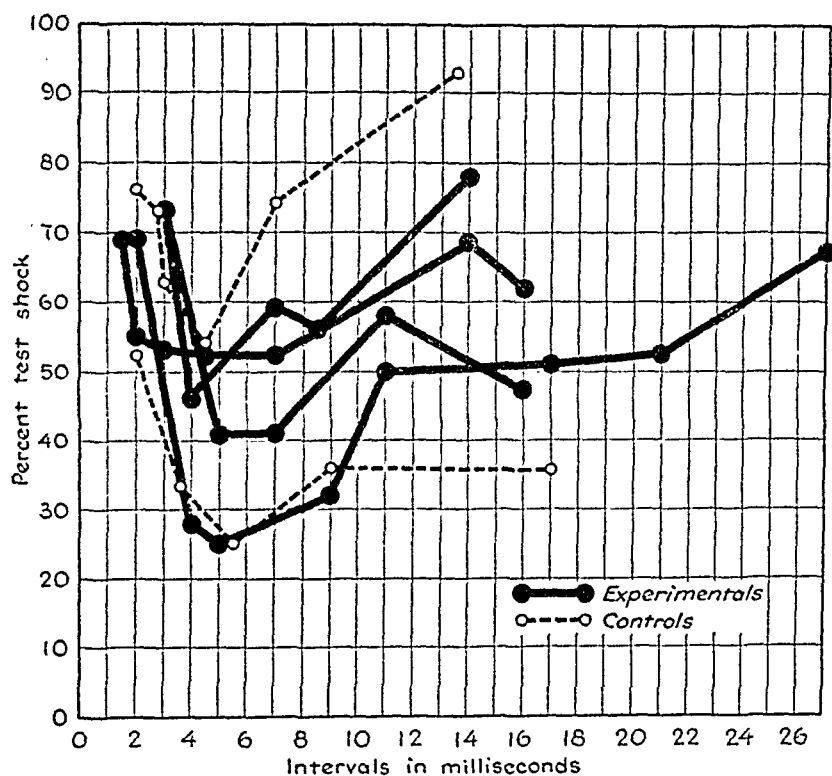


Fig. 2. Condition curves of 2 normal and 4 chromatolytic sides.

cord. Hence, only evidence of a suggestive nature is presented to implicate the recurrent collaterals as mediators of conditioning impulses.

#### SUMMARY

The conditioning of a proprioceptive discharge by an antidromic volley, in closely related groups of spinal motoneurons, was not altered by chromatolysis and the subsequent reduction of the external action potential of the conditioning motoneurons. This is direct evidence that the external action potential of the antidromically fired nerve cells does not exert a demonstrable conditioning effect on closely related groups of spinal motoneurons. This also suggests that the recurrent collaterals may be implicated as mediators of conditioning impulses.

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# EFFECT OF CORTICAL STIMULATION ON RESPIRATORY RATE<sup>1,2</sup>

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**D**URING the course of an investigation of cortical autonomic representation in dogs and cats (1), respiratory changes were observed almost invariably in association with autonomic responses. Because of the possible reflex effects of respiratory alterations on the autonomic functions under consideration (e.g. gastrointestinal motility) respiration was recorded in all experiments. With the appearance of the phenomena to be described, it became necessary to carry out a separate study designed to elucidate the effect of various anesthetic agents and stimulus characteristics on cortically induced respiratory changes, as well as to determine the cortical respiratory areas and the types of responses capable of being produced therefrom.

The literature on respiratory responses from the lateral and orbital surface has been reviewed by Delgado and Livingstone (2) recently and the reader is referred to their table 1 for a summary. There is general agreement among the recent workers that there is an area for acceleration of respiration in the sensory-motor cortex, and an area for inhibition and arrest on the orbital surface. Recently both acceleration and arrest of respiration have been reported from the cingulate gyrus (3).

## METHODS

Experiments were performed on 19 dogs and 4 cats, all adults. Anesthesia was obtained as follows: *dogs*: 7-chloralose and urethane (1:10), 5-nembutal, 4-dial, 3-pentothal; *cats*: 3-dial, 1-2 per cent novocaine locally. Thoracic and abdominal excursions were recorded by a closed air system from a balloon girdle or rubber bellows apparatus attached outside the thorax and abdomen. Diaphragmatic movements were recorded from a balloon placed high up in the fundus of the stomach under the diaphragm. To eliminate abdominal wall artefact in the records of diaphragmatic respiration, the abdomen was incised widely transversely, the intestines retracted in a moist towel, and the temperature of the whole kept constant at 38-40 degrees. Blood pressure and pulse rate were also recorded, as well as many simultaneous records of gastrointestinal movements. All records were preserved on a continuous ink-writing kymograph.

The orbital surface was exposed by removal of the orbital contents. The cingulate gyrus was exposed by clipping or coagulation of the veins entering the longitudinal sinus, and retraction or removal of the hemisphere on the same side. This provided an adequate exposure of the opposite cingulate gyrus. The cortex was stimulated with bipolar silver wire electrodes of 2 to 3 mm. separation held in a ball-and-socket clamp which permitted stability and easy mobility as desired. The points stimulated were recorded on a separate brain chart, or by photographing numbered tags placed directly on the cortex.

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<sup>3</sup> N.R.C. (Can.) Fellow in Medicine 1947-48.

The stimulus was of the square wave type with independently variable frequency, intensity and wave duration to the extent that each wave could occupy 80 per cent of the interval between successive waves without distortion. An exploratory stimulus of 1 to 6 volts, 60 cycles and 6.4 milliseconds was used, since it was found to give most consistent results.

### RESULTS

*Chloralose and Urethane.* The predominant response from stimulation of the cortex of dogs under chloralose and urethane was acceleration of respiratory rate. This effect was produced most readily from a point about 5 mm. anterior to the lateral end of the cruciate sulcus (fig. 1*A*). Less marked responses could, however, be

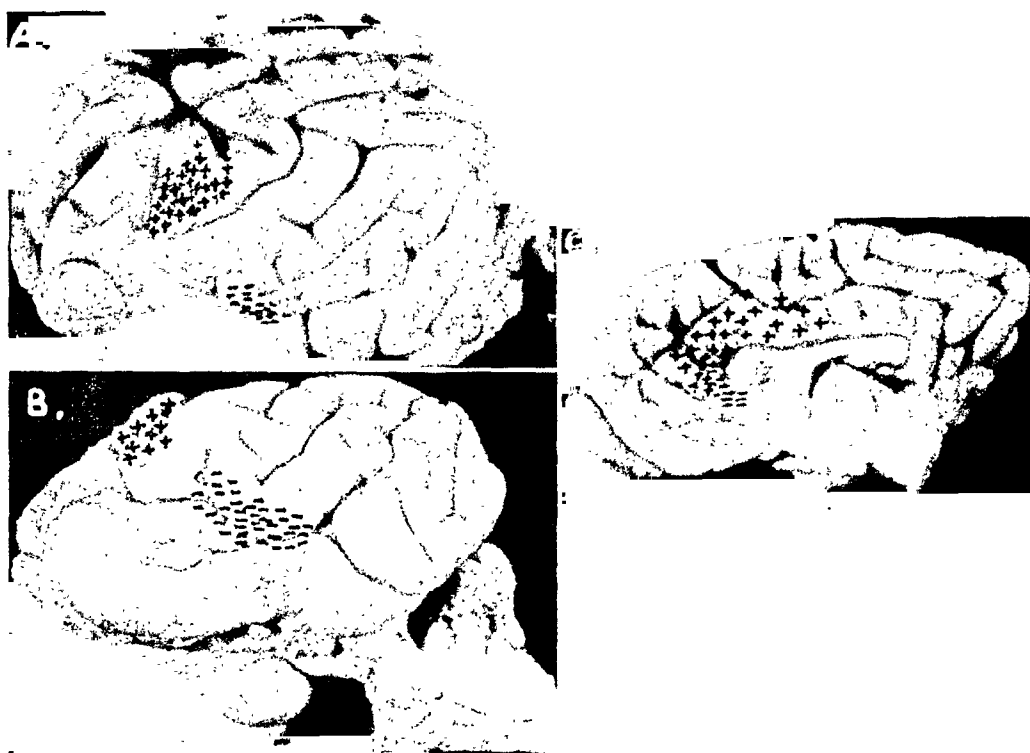


Fig. 1. DOG BRAIN. Alterations of respiratory rate from cortical stimulation under barbiturate narcosis or local anesthesia. *A*, lateral surface; *B*, orbital surface; *C*, medial surface. + = respiratory acceleration; - = respiratory slowing or arrest.

produced from the anterior and posterior sigmoid gyri, the anterior end of the coronal, ectosylvian and sylvian gyri and an irregular strip of cortex anterior to the presylvian sulcus and lateral to the olfactory tract. Medially, acceleration occurred from stimulation of the entire middle and anterior portions of the cingulate gyrus.

Slowing of respiration using chloralose and urethane was seen in only two experiments, the area for the response being the anterior end of the ectosylvian gyrus (fig. 1*B*). Arrest of respiration was never seen in the dog under this type of anesthesia. No inhibitory effect on respiration could be obtained from the sub-genual portion of the anterior cingulate gyrus with this anesthetic.

A heretofore unreported phenomenon was produced at a very light level of chloralose and urethane anesthesia (fig. 2). This consisted of an increase of rate, a

diminution of the thoracic excursion and an increase in the diaphragmatic excursion during stimulus. Although the stimulation lasted only 10 to 20 seconds, there was a gradual decrease of the thoracic excursion until in 10 to 100 seconds the thorax came to rest in the expiratory position, and the diaphragm alone continued to perform the respiratory act. Observation of the animal at this time showed completely flaccid thoracic and abdominal walls, the abdomen moving only passively due to the diaphragmatic contractions.

The full 'shift' persisted for 30 to 60 seconds when, by the same gradual process, thoracic respirations gradually increased in amplitude and the diaphragmatic ex-

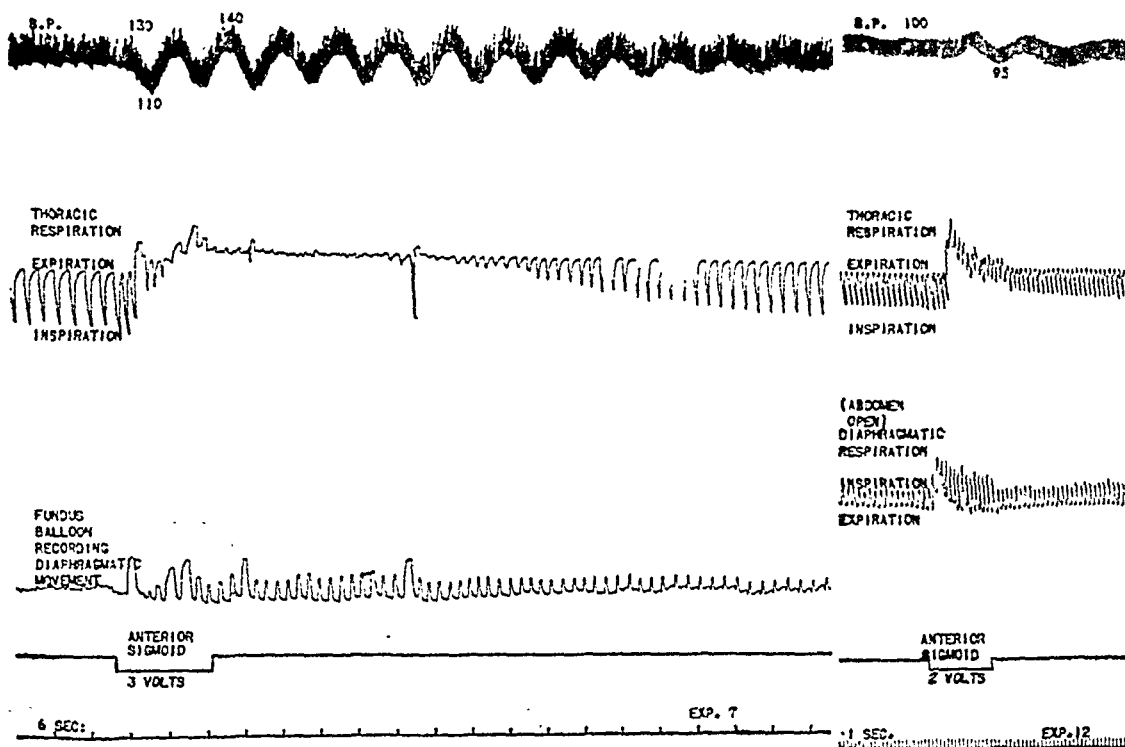


Fig. 2 (*left*). 'SHIFT' OF RESPIRATION from predominantly thoracic to predominantly diaphragmatic, from stimulation of the lateral end of the anterior sigmoid gyrus.

Fig. 3 (*right*). SLIGHT ACCELERATION OF RESPIRATION with decrease of thoracic excursion and increase of diaphragmatic excursion, but without complete 'shift' from same point as figure 2 with weaker stimulus.

cursions diminished until the original conditions were reestablished 2 to 3 minutes after stimulation. This response could be reproduced at will from those areas of the cortex which gave an augmentation of respiration in any given experiment. It was also elicited (as were the other changes herein described) as a reflex response to certain types of peripheral stimulation.

The chief factor in the production of this phenomenon was a light level of chloralose and urethane anesthesia. Deepening the anesthesia or reducing the strength of the stimulus eliminated the second part of the response, so that slight acceleration with diminution of thoracic excursion and increase of diaphragmatic excursion occurred, and was confined to the period of actual stimulation; the 'after effect' did not appear (fig. 3).

**Barbiturates.** The depression of autonomic reflexes produced by the dosage of barbiturates sufficient to inhibit somatic reflexes seemed to be much greater than that resulting from similar dosage of chloralose and urethane. Nevertheless, the following responses were not specifically due to effects of anesthesia since they could be produced from the same areas in unanesthetized preparations.

The changes in respiratory rate from cortical stimulation were qualitatively similar for all the barbiturates and are summarized in figure 1. With this group of anesthetic agents there are four well defined cortical areas from which changes in respiratory rate may be produced:

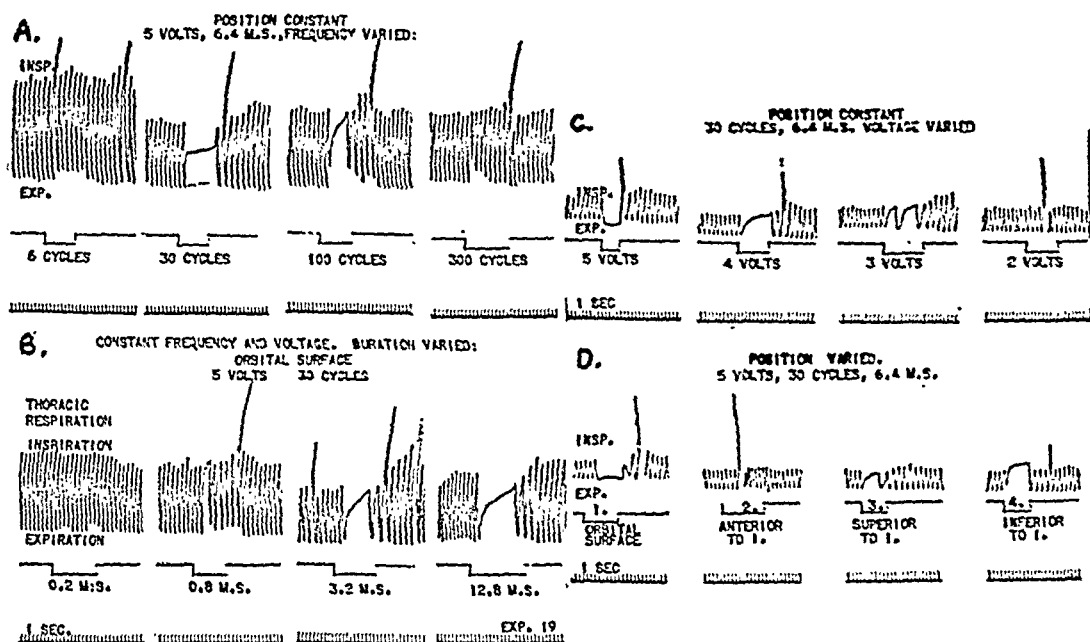


Fig. 4. ANALYSIS OF STIMULUS CHARACTERISTICS for respiratory inhibition from the insular-orbital region. A, frequency varied; B, wave duration varied; C, intensity varied; D, position altered. Note apparent acceleration following gap in D-2.

**Acceleration:** a) centering around the lateral end of the anterior sigmoid gyrus and extending anteriorly to and across the presylvian sulcus (fig. 1A); b) the middle and most of the anterior portions of the cingulate gyrus, with the exception of b below (fig. 1C).

**Inhibition:** a) roughly defined as the 'insular-orbital region'<sup>4</sup> which includes the anterior ends of the sylvian and ectosylvian gyri (anterior composite gyrus) and the adjacent parts of the frontal lobe and olfactory tract (fig. 1B); b) a small area on the anterior cingulate gyrus anterior and inferior to the genu of the corpus callosum (fig. 1C).

Using a higher speed kymograph it could be shown that the latent period for

<sup>4</sup> This term is used to emphasize the homology between the sylvian and anterior sylvian gyri of animals and the anterior part of the Island of Reil in man (4). While the entire area is in direct relationship to the roof of the orbit, the term 'orbital surface' should be reserved for the orbital gyri and other portions of the inferior surface of the frontal lobe.

these responses was dependent upon the phase of the respiratory excursion during which stimulation began. For example, inhibition in the cat, occurring maximally in expiratory arrest (fig. 4C), appeared only at the conclusion of the respiratory cycle during which the stimulus was applied. The alteration of rate could be maintained for a variable period, depending on its intensity. Complete arrest was seldom prolonged beyond 10 seconds, when escape occurred at a slower rate and diminished amplitude. With cessation of stimulation, the original rate was usually resumed immediately, although there was occasionally a short after effect.

*Stimulus Characteristics.* Acceleration responses were more readily produced in the dog, while inhibition was most consistently apparent in the cat. For this reason the following analysis of the quantitative and qualitative effect of stimulus characteristics on the cortically induced respiratory inhibition was carried out on the cat. Optimal frequencies lay between 30 to 60 cycles per second (fig. 4A), while above 200 cycles per second and below 10 cycles per second there was either no change or, occasionally, slight acceleration. Optimal wave duration with this type of stimulator was 6.4–12.8 m.s. with an effective range of 3.2–25.4 m.s. (fig. 4B). Beyond that range the inhibition was submaximal, but acceleration never appeared as long as the intensity and frequency were not altered. The phase during which stimulation was applied bore no relation to the phase of arrest. By altering the intensity it was possible to show that the maximal response consisted, in the cat, of arrest in expiration (fig. 4C) due to inhibition of the inspiratory cycle. The same method could be used to determine the 'center' of the responsive area (fig. 4D).

A similar analysis of the stimulus characteristics for acceleration revealed wider variations of the same order as for inhibition. However, while it was occasionally possible to produce acceleration from the so-called inhibitory areas using stimuli outside the mentioned ranges or very light anesthesia, it was not possible to produce respiratory arrest from the so-called accelerator areas.

*Blood Pressure.* Blood pressure responses were not studied independently of respiration but an analysis of all the changes in this series with chloralose and urethane anesthesia showed that the usual response was depressor from all the respiratory areas. This was usually more marked when there was an alteration of respiration and seemed to be greatest from the sub-genua portion of the anterior cingulate gyrus, where the fall sometimes amounted to 60 mm. On the other hand, with the barbiturates there was frequently a slight rise of the blood pressure at the beginning of stimulation (particularly from the orbital surface), followed by a somewhat greater fall if respiratory inhibition occurred, and finally a slight rise following the end of the stimulus, before the original conditions were reestablished. Since these alterations were more marked when there were also respiratory changes, it was felt that they were at least partly cardio-respiratory reflexes, rather than indicative of specific cardio-vascular centers.

It is interesting in this regard that the blood pressure response from the anterior cingulate gyrus could be reversed by cutting the vagi. Prior to bilateral vagotomy a fall of 30 mm. occurred from stimulation of this area, whereas after vagotomy and the introduction of artificial respiration a rise of 30 mm. was produced by stimulation of the same point.

## DISCUSSION

The influence of different anesthetic agents on respiratory responses from the cortex was first discussed by Spencer (5) who felt that the level of anesthesia, as well as the type of anesthetic used, determined the quantitative and, to some extent, the qualitative variations in results. The results presented here bear out this criticism of much of the past and current work in the field of autonomic responses from the cortex. It must be concluded that no blanket statement can be made regarding the type of response obtainable from any given cortical area, unless and until the effects of the anesthetic agents on the cortical and subcortical neuronal mechanisms, and the effector organs, are known. Similarly, full account must be taken of the interactions of the various changes in different systems (e.g. B.P. and respiration) which are altered by stimulation of the same area. To a lesser extent we must also begin to appreciate the influence of variations in stimulus characteristics.

We have confirmed previous workers regarding the presence of respiratory acceleration area about the lateral end of the anterior sigmoid gyrus and presylvian sulcus, and an inhibitory area in the 'insular-orbital' region (not from the coronal gyrus, 6). The respiratory responses from the cingulate gyrus have been clarified. The latter has been divided into two functionally distinct portions, a large area for acceleration and a small, well localized area anterior and inferior to the genu of the corpus callosum for inhibition.

It has been shown that inhibition of respiratory rate may be produced by stimulation of the 'insular-orbital' surface in unanesthetized animals, similar in every respect to that occurring in animals under barbiturate narcosis. Therefore, the absence of 'inhibitor' area in dogs under chloralose and urethane is due to the level of anesthesia or some other action of these agents on the neuronal mechanisms involved.

The previously unreported phenomenon consisting of a shift from thoracic to diaphragmatic respiration similarly must be considered to be a non-specific response, since it was obtainable from any cortical respiratory accelerator area under the required conditions of anesthesia. It is not unlike the normal respiration in the relaxed and weary state, the respiration of deep sleep, or that of third stage anesthesia. It differed from the usual cortical motor responses in that it continued despite cessation of the stimulus. It was occasionally preceded by apparent hyperventilation, produced either by increased rate or depth of respiration, but could not be produced by hyperventilation alone. The importance of this phenomenon, then, lies not in the cortical area from which it can be induced (since this is diffuse) but in the fact that a disturbance of the balance between the two major effector muscle groups for the respiratory act can be produced by cortical stimulation.

It was not within the scope of this work to analyze the other properties of the respiratory act, such as inspiratory and expiratory 'tonus' (5) nor to make a detailed study of the variations of amplitude of the abdominal and thoracic components. Examples of these may be seen in figures 3 and 4. It is obvious, however, that these factors play a rôle equal to, if not greater than, the rate in the volume of respiratory exchange and will require eventual elucidation.

The effect of different frequencies of stimulation on respiratory responses induced by direct vagal stimulation has been reported (7, 8).

The analysis of the stimulus characteristics is not yet complete. However, the results presented indicate that there are limits to the electrical characteristics of the stimulus beyond which the cortical mechanisms will not respond. This will find explanation eventually in the electrical properties of the individual neuronal pathways. It may be, and we have suggestive evidence, that there are different parameters of electrical stimuli for different systems represented in the same cortical area.

Recently it has been suggested that an opposite effect on the same function may be produced by different frequencies of stimulation on the same cortical area (2). Although we have also seen occasional acceleration from the inhibitory areas of the orbital surface with barbiturates, this has not been consistently true. It seems more likely that this is what Wyss has called a non-specific response (8). However, as indicated by the reversal of blood pressure response to stimulation of the sub-genual portion of the anterior cingulate gyrus after bilateral vagotomy, it may be that there is a double representation of both sympathetic and parasympathetic systems in the one region (9). When the dominant system is abolished the opposite effect appears.

All the respiratory responses obtained by cortical stimulation can be obtained through peripheral reflex mechanisms (10). Arrest of respiration from the insular orbital region and from the sub-genual portion of the anterior cingulate gyrus is analogous to that obtained by vagal stimulation, through medullary centers. Acceleration from the anterior sigmoid and anterior and middle cingulate gyri is similar to that produced by stimulation of peripheral noci- and chemoreceptors. Thus it is very probable that the cortical representation of respiration is simply a more specialized reduplication of lower level mechanisms, presumably allowing of more elaborate and integrated control.

The similarity of the 'vagal' motor effects on gastric motility and blood pressure from stimulation of the insular-orbital surface and the sub-genual portion of the anterior cingulate gyrus has been discussed in another paper (1). The addition of similar respiratory effects from the two areas is further evidence of a functional relationship between them.

#### SUMMARY

The effect of different anesthetic agents and stimulus characteristics on changes of respiratory rate by electrical stimulation of the cortex has been considered.

There are two areas for inhibition of respiratory rate in the dog and cat: *a*) a small area on the anterior part of the cingulate gyrus just anterior and inferior to the genu of the corpus callosum; *b*) a larger area designated the 'insular-orbital' region which includes the anterior part of the anterior sylvian and ectosylvian gyri, the posterior portion of the frontal lobe between the presylvian and anterior rhinal fissures, and the adjacent portion of the olfactory tract.

Acceleration of respiration in the dog and cat may also be produced from two separate areas: *a*) the anterior sigmoid and presylvian region; *b*) the anterior and middle portions of the cingulate gyrus, exclusive of the sub-genual area mentioned above.

The maximal stimulus characteristics for inhibition of respiratory rate from the

insular-orbital surface using a square wave stimulator have been outlined. A previously unreported phenomenon consisting of shift from predominantly thoracic to predominantly abdominal respiration is discussed, and a functional relationship between the insular-orbital surface and the sub-genual portion of the anterior cingulate gyrus is suggested.

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# VARIATION IN ACETYLCHOLINE CONTENT OF THE BRAIN WITH PHYSIOLOGICAL STATE

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**A** CETYLCHOLINE is present in significant quantity in the mammalian brain. It appears to occur mainly in the form of a physiologically inactive complex which liberates free acetylcholine when the tissue is treated with extractants. The level in the brain *in vivo* must depend on *a*) the rate of synthesis, *b*) the rate of liberation from the complex and *c*) the rate of loss by enzymic breakdown or by diffusion from the tissues.

It has been suggested that acetylcholine may play a part in nervous transmission in the central nervous system. If nervous activity is associated with an increased liberation and breakdown of acetylcholine, the level in the brain *in vivo* might be expected to vary to some extent with the state of functional activity of the brain. The level should be maximal in conditions of reduced activity, as in anesthesia and sleep, while it might show a temporary fall in states of increased neuronal activity, as during convulsions. The present investigation was carried out to test if evidence could be found of any variation of the acetylcholine content with the degree of functional activity of the brain.

## METHODS

Acetylcholine determinations were carried out on the whole brains of young Wistar albino rats of 20 to 30 gm. The animals were killed by immersion in liquid air, which produced a rapid fixation of any biochemical changes in the tissues and minimized post-mortem changes due to the breakdown or resynthesis of acetylcholine in the brain. This method of killing also appeared to cause less stimulation of the brain than other methods such as decapitation (1). The liquid air was put in a large wide-mouthed beaker so that the animals could be dropped straight in without delay.

Series of animals were taken for acetylcholine estimation: *a*) during anesthesia; *b*) while sleeping; *c*) in the normal waking state; *d*) during emotional excitement *e*) after electrical stimulation of the brain; and *f*) during convulsions.

Anesthesia was obtained by intraperitoneal injection of sodium pentobarbital (5 mg/kg.); the temperature of the animals was maintained by placing them on a warm metal plate. Animals were made to sleep by the warmth and light of an operating table lamp placed over the cage. Emotional excitement was produced by tipping from side to side in a large beaker for 4 minutes. Electrical stimulation of the brain was effected with 35 to 50v. A.C. at 50 cycles/second by platinum electrodes of 0.25 sq. cm. area applied to the shaved scalp 0.5 cm. posterior to the eyes. Electrical stimu-

lation for 1 to 3 seconds produced satisfactory convulsions after a usual latent period of about 10 seconds, during which the animals were in coma. The procedure was similar to that used in the electroshock treatment of psychiatric patients. Further details of these methods are given by Richter and Dawson (2, 3). A normal control was included with one from each experimental group in any particular series of determinations and littermates were used as far as possible.

*Extraction of Acetylcholine.* The validity of the results depended on obtaining a consistently high extraction of the total brain acetylcholine. The reliability of the usual methods has sometimes been questioned and special attention was therefore given to the method of extraction. The brain was dissected from the skull using chilled instruments and keeping the brain frozen by the use of further quantities of liquid air. It was then finely powdered in a cooled steel crusher. In order to minimize any errors due to incomplete extraction two different methods were used.

*Method 1. Extraction with Buffered Saline.* In this method the powdered brain was stirred at 0° C. into a mixture of 1 ml. acetate buffer pH 4.0 (made by diluting 100 ml. N acetic acid and 31 ml. N sodium hydroxide solution to 700 ml.) and 3 ml. eserized acidified amphibian Ringer-Locke solution. The Ringer-Locke solution before acidification contained 6.5 gm. NaCl, 0.14 gm. KCl, 0.2364 gm.  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  and 0.2 gm.  $\text{NaHCO}_3$  per liter; it was brought to pH 4.0 with N/4.5 hydrochloric acid, using bromophenol blue, and 1 part in 4000 of eserine sulfate was added. The solution in a centrifuge tube was weighed before and after addition of the powdered brain. The mixture was rapidly brought to the boil on a Bunsen flame, allowed to cool, stirred with a glass rod and centrifuged for 5 minutes. The supernatant solution was decanted, the residue washed with 1 ml. eserized amphibian Ringer-Locke solution at pH 4 and the combined supernatant solutions were kept in stoppered graduated tubes in the refrigerator until required for assay.

*Method 2. Extraction With Trichloracetic Acid.* In this method the powdered brain was stirred with 4 ml. 10 per cent trichloroacetic acid at 0° C. in a previously weighed centrifuge tube and the mixture was shaken thoroughly. The tubes were kept on ice for 30 minutes, the mixture was then centrifuged 4 minutes, the residue washed with 1 ml. 10 per cent trichloroacetic acid and the combined supernatant solutions brought to pH 4 with N sodium hydroxide solution. The solution was kept in the refrigerator until assayed. The presence of sodium trichloroacetate at the dilution used did not interfere with the estimation of acetylcholine by the frog rectus preparation.

In 6 experiments the powdered brain was ground with sand in a cooled mortar containing the extractant at 0° C.; this modification of the procedure produced no significant difference. In a further series of 9 animals the completeness of the extraction was tested by re-extracting the residue from the first extraction. Assays carried out on the second extract, of which 5 were made with buffered saline and 4 with trichloroacetic acid, gave a further small amount of acetylcholine; the figures were 0.04, 0.02, 0.01, 0.0, 0.08, 0.0, 0.04, 0.02 and 0.0  $\mu\text{g/gm.}$  acetylcholine. These figures indicated that the first extract, made by either *method 1* or *method 2* contained 95 to 100 per cent with a mean of 98 per cent of the total acetylcholine obtained in the two extractions. Further tests showed a high recovery of known amounts of acetylcholine added to the residue and then extracted by the normal procedures. In 6

experiments the amounts added were 2.0, 2.0, 1.0, 2.0, 2.0 and 1.0  $\mu$ g. acetylcholine; the amounts found by assay on extraction with buffered saline were 1.85, 1.9 and 0.95  $\mu$ g. in the first 3 experiments and 2.0, 1.9 and 0.97  $\mu$ g. on extraction with trichloroacetic acid in the last 3 experiments, respectively. The close agreement in the figures obtained for the brain acetylcholine by the two different methods of extraction also gave evidence that the methods of extraction used were satisfactory (table 2).

*Acetylcholine Assay.* In a few preliminary experiments the leech muscle and cat blood pressure preparations were used, but in all the subsequent work the frog rectus method was preferred as it was apparently more satisfactory for the brain extracts which had to be tested. The frog rectus muscle was set up in a 4 ml. bath of amphibian Ringer-Locke solution. It was allowed to stand 2 hours in the oxygenated solution under a small tension of 0.75 gm. The solution was frequently changed during the first hour and for the second hour it was replaced by Ringer-Locke solution containing 1 in 100,000 eserine sulfate. Before starting the determinations the muscle was tested several times with a small amount of acetylcholine to make sure that its sensitivity was constant and that it always relaxed to the same length. If these conditions were not fulfilled the muscle was left for a further 30 minutes in eserinated Ringer-Locke solution and then re-tested. Most of the assays were carried out in an unheated laboratory during the winter months, but if the room temperature rose above 10° C. the bath temperature was reduced to 10° before use.

Immediately before assay, each brain extract was divided into two equal parts, A and B. Solution A was brought to pH 7 with sodium hydroxide solution and then diluted with amphibian Ringer-Locke solution so that each ml. contained the extract from 100 mg. of brain. Solution B was made alkaline (pH 11) with sodium hydroxide solution, boiled to destroy the acetylcholine, brought to pH 7 and diluted with Ringer-Locke solution to the same volume as solution A. Portions containing 1 ml. of solution A made up to 4 ml. with Ringer-Locke solution were then assayed against known amounts of acetylcholine chloride made up in 1 ml. of solution B and similarly brought to 4 ml. with Ringer-Locke solution. This method, due to Feldberg (4), avoided errors due to sensitizing substances present in the brain extracts. Acetylcholine solutions were made up daily from sealed phials containing 1 mg. acetylcholine in 1 ml. 10 per cent dihydrogen sodium phosphate solution. The eserinated Ringer-Locke solution was made daily by adding the calculated amount of solid eserine sulfate to a diluted stock solution.

By the described procedure the brain from a 30-gm. rat provided sufficient extract for 5 contractions of the rectus muscle and it was possible to bracket one or more of these between two closely approximating amounts of acetylcholine. The sensitivity of the rectus preparation under the conditions used was such that it would regularly detect as little as 0.02  $\mu$ g. of acetylcholine in the 4 ml. bath; it would detect differences of the same order, so that estimations of acetylcholine content could be made with an error of not more than 10 per cent. Results are expressed as  $\mu$ g. acetylcholine chloride per gm. fresh brain tissue.

## RESULTS

*Preliminary Experiments.* The substance present in brain extracts which causes contraction of the frog rectus muscle was shown to have the properties of acetyl-

choline in that: *a*) it was destroyed by heating with alkali at pH 11.0 for 1 minute; *b*) it was destroyed by incubating with cholinesterase prepared from red blood cells; *c*) it caused contraction of the leech muscle; and *d*) it produced a fall in the blood pressure, abolished by atropine, in the cat.

In a series of preliminary experiments, which are described in brief, the brain acetylcholine content of rats of 40 to 50 gm. was estimated with the leech muscle preparation. The acetylcholine was extracted with buffered saline (*method 1*) and assayed in the usual manner against a standard solution of acetylcholine in saline. In a further series of experiments carried out in collaboration with Dr. F. C. MacIntosh and Dr. R. M. C. Dawson the acetylcholine in extracts prepared with trichloroacetic acid (*method 2*) was estimated with the cat blood pressure preparation. Substances other than acetylcholine in the brain extracts tended to interfere with the acetylcholine response and, as in these preliminary experiments the animals taken during

TABLE 1. VARIATION IN ACETYLCHOLINE CONTENT OF RAT BRAIN

METHOD OF ESTIMATION	ANESTHESIA	SLEEP	NORMAL	EXCITED	CONVULSIONS
	$\mu\text{g}/\text{mg.}$	$\mu\text{g}/\text{mg.}$	$\mu\text{g}/\text{mg.}$	$\mu\text{g}/\text{mg.}$	$\mu\text{g}/\text{mg.}$
Leech muscle	2.9	2.22	1.9	1.4	1.7
	2.4	2.6	1.6	1.5	1.1
	2.8	2.3	1.3	1.6	1.1
			1.4		1.8
Mean	2.7	2.4	1.55	1.5	1.4
Cat blood pressure	2.8		1.7	2.0	1.6
	2.5		2.5	2.1	1.4
	2.2		1.8	1.9	1.5
			2.4	1.5	
			1.5		
Mean	2.5		2.0	1.9	1.5

convulsions were transferred to liquid air during the first 5 to 10 seconds of the convulsions, the maximum fall was missed; but by both methods of assay the relative acetylcholine values showed a general trend towards lower acetylcholine levels in states of greater activity (table 1). The acetylcholine content for anesthetized animals was 70 to 100 per cent higher than for animals taken during convulsions.

*Normal Series.* The acetylcholine content of the brains of 15 young rats of 20 to 30 gm., determined by the frog rectus method, gave a mean value of 1.25  $\mu\text{g}/\text{gm.}$  fresh brain tissue, with a range of 0.9 to 2.0  $\mu\text{g}/\text{gm.}$  and S.D. of  $\pm 0.29$ . This mean value was lower than some other figures given in the literature and lower than the mean for the preliminary experiments. This may be due in part to the special precautions taken to prevent the resynthesis of acetylcholine, which is rapid in nervous tissue (5), and in part to the use in these experiments of younger animals: it was shown by Welsh and Hyde (6) that the acetylcholine content of the rat brain increases with age. The method of assay which was used corrected for the presence of substances in the brain extracts which sensitize the rectus muscle to acetylcholine and

which tend to give unduly high figures for the acetylcholine content if they are not taken into account. The effect of such sensitizing substances varies with the particular brain extract and with the assay preparation, but it was found that under the conditions of these experiments the figure obtained for the acetylcholine content could be as much as 50 per cent above the true value if this source of error was ignored. Good agreement was obtained between the groups in animals in which the acetylcholine extraction was carried out by different methods (table 2).

Animals in the normal series were brought into the laboratory some time before killing and kept in a warm place. After a few hours under these conditions they be-

TABLE 2. ACETYLCHOLINE CONTENT OF RAT BRAIN IN DIFFERENT STATES OF ACTIVITY. FROG RECTUS METHOD

EXTRACTANT	ANESTHESIA	SLEEP	NORMAL	EXCITED	ELECT. STIMUL.	CONVULSIONS
	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$
Buffered saline	2.1	1.5	1.3	0.95		0.55
	1.9	1.3	1.3	0.75		0.40
	1.6	1.6	1.3	0.85		
			1.0	1.3		
			1.2	1.0		
				1.2		
				1.1		
Mean	1.87	1.47	1.22	0.99		0.48
Trichloroacetic acid	1.5	1.4	1.0	0.90	0.45	0.55
	1.9	1.8	2.0	0.85	0.50	0.50
	1.8	1.5	1.5	0.55	0.80	0.60
	1.9	1.0	1.2	0.53	0.55	0.68
	1.4		1.4	0.80	0.65	0.50
			0.95	1.3	0.30	
			1.5	0.95	0.65	
			1.0	0.40	0.65	
			0.9	0.60	0.42	
			1.2	0.84		
Mean	1.7	1.43	1.27	0.82	0.55	0.57
Overall mean	1.76	1.44	1.25	0.87	0.55	0.56

came quiet and they were transferred to liquid air while feeding or while moving quietly in their cage. The liquid air was contained in a wide-mouthed beaker and usually they entered it without any sign of being frightened, but any animal which was felt to struggle before entering the vessel was discarded. These precautions may account for the small standard deviation found in these experiments.

*Effects of Anesthesia and Sleep.* Eight animals lightly anesthetized with sodium pentobarbital gave a mean acetylcholine content of 1.76  $\mu\text{g./gm.}$  (range 1.4-2.1; S.D. 0.24) and 7 animals taken in the sleeping state gave a mean of 1.44  $\mu\text{g./gm.}$  (range 1.0-1.8; S.D. 0.25). Both of these values were higher than for normal animals and the differences were statistically significant ( $P < 0.01$ ); they were also significantly differ-

ent, at the 0.05 level of probability, from one another. The result for anesthetized animals agrees with a previous report of Tobias, Lipton and Lepinat (7), who also found a higher brain acetylcholine content in anesthetized rats. The individual values obtained by them in both groups were somewhat higher than those obtained in the present series, but this may be due to differences in the experimental conditions and technique.

*Emotional Excitement, Electrical Stimulation and Convulsions.* A series of 17 rats, which had been excited for 4 minutes by the method of repeatedly removing their support, gave a mean brain acetylcholine content of  $0.87 \mu\text{g}/\text{gm}$ . (range  $0.4-1.3$ ; S.D.  $0.26$ ). This was significantly lower than the normal mean. Lower values were also obtained for further series of animals taken during electrical stimulation of the brain

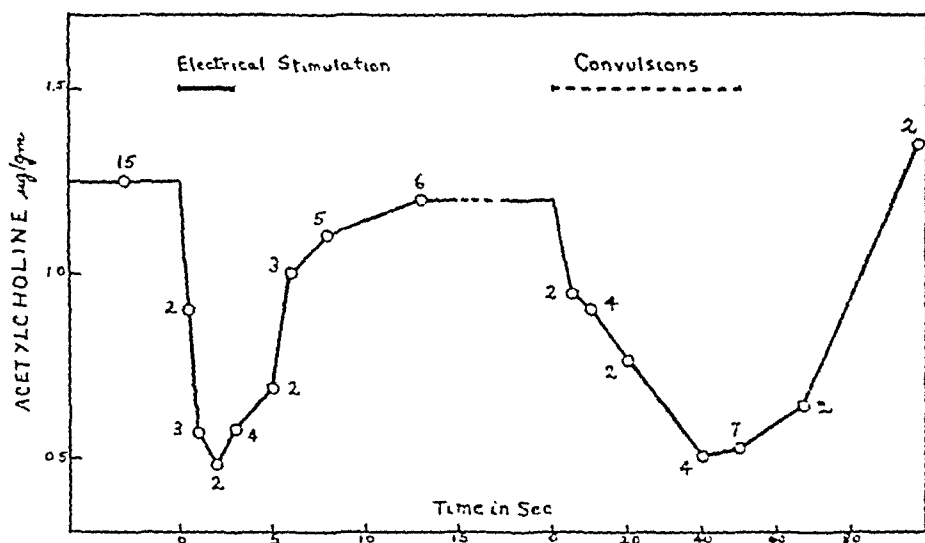


Fig. 1. SHOWING EFFECT OF ELECTRICAL STIMULATION and subsequent convulsions on the acetylcholine content of the rat brain. Each point gives the mean value for 2 or more animals, the number of which is given alongside. Electrical stimulation was given for 3 sec. except for animals killed after a shorter period of stimulation. The latent period before the commencement of convulsions varied from 8 to 15 sec. for different animals. The values during and after convulsions are therefore given on a separate time scale starting at the time when convulsions commenced.

and during convulsions. The differences from the normal mean were significant in each group at the 0.01 level of probability. The electrically stimulated animals, which were transferred to liquid air after passage of the electric current through scalp electrodes for 1 to 3 seconds, gave a mean acetylcholine content of  $0.55 \mu\text{g}/\text{gm}$ . with a range of  $0.30$  to  $0.80$  and S.D.  $0.16$ . The electrical stimulation under these conditions caused an immediate generalized muscular spasm, followed by a period of coma lasting for about 10 seconds. Convulsions usually started then and continued for about 40 to 50 seconds. The convulsions gradually became intermittent and then, often after a period of hyperactivity, the animals became relatively quiet and inactive. Animals taken after the convulsions had continued for 40 to 50 seconds gave a mean brain acetylcholine content of  $0.56 \mu\text{g}/\text{gm}$ . with a range of  $0.4$  to  $0.68$  and S.D.  $0.09$ .

*Rate of Loss and Resynthesis of Acetylcholine in the Brain In Vivo.* In order to study the rate of the change in the brain acetylcholine level, groups of animals were

taken for examination at various times during and after electrical stimulation and during the convulsions which ensued. The results for 65 animals, which are given diagrammatically in figure 1, showed that the sharp fall in the brain acetylcholine level during electrical stimulation was followed by a rapid rise. In animals taken after 1 to 3 seconds of electrical stimulation there was a loss of over 50 per cent of the total brain acetylcholine content. The times given in figure 1 refer to the time when the animal was transferred to liquid air. The transference, timed with a stop-watch, took 0.4 to 0.6 seconds. The time taken for the cortex of the brain to cool to 0° C., shown by means of a thermocouple, was approximately 4 seconds. A part of the acetylcholine breakdown might have occurred during the few seconds of cooling, but the values obtained in the few seconds immediately after electrical stimulation showed a rapid return to the normal level, indicating that under these conditions resynthesis of acetylcholine was also rapid. Even if anoxia occurred, the brain would contain ample phosphocreatine and adenosine triphosphate to enable resynthesis of acetylcholine to take place. It is therefore possible that resynthesis proceeded during the 4 seconds of cooling, so that the acetylcholine content of the brain during electrical stimulation was actually lower than would appear from the figures obtained. From the slope of the resynthesis curve a rough estimate of the rate of acetylcholine synthesis in the brain *in vivo* could be made: the calculated rate was 7  $\mu\text{g/gm.}$  fresh brain tissue/minute.

The onset of convulsions was associated with a second fall in the acetylcholine content, which fell more gradually to 50 per cent of the normal value in 40 to 50 seconds. After the convulsions were over, the acetylcholine level again began to rise, but at this stage some of the animals were in an excitable state and the return to normality in the brain acetylcholine level was more gradual than immediately after electrical stimulation.

*Chromodacryorhesis.* It was noted that chromodacryorhesis ('bloody tears') occurred after electrical stimulation by scalp electrodes under the conditions described. Since it occurred only when the stimulation was sufficient to cause coma and not when the shock was insufficient to produce this effect, it should probably be attributed to the central stimulation rather than to a local action. The observation appears relevant since it is known that chromodacryorhesis is produced by injection of a small amount of acetylcholine (2.0  $\mu\text{g/kg.}$ ) in the rat, and it has been regarded as an indicator of a release of acetylcholine into the general circulation (8).

#### DISCUSSION

Evidence is presented that the acetylcholine content of the rat brain, determined after rapid fixation by freezing with liquid air, varies with the physiological state of the animal. The level was significantly raised in animals taken during anesthesia and during sleep; it was significantly lowered in animals taken during emotional excitement, after electrical stimulation of the brain and during convulsions. The acetylcholine level thus appeared to vary inversely with the degree of functional activity of the brain. The observed changes were relatively large. Electrical stimulation for 1 to 3 seconds caused a loss of over 50 per cent of the whole brain acetylcholine. The level was raised in anesthesia 40 per cent above the normal value and more than 300 per cent above that of animals taken during convulsions. Studies of the rate of

loss and resynthesis of acetylcholine *in vivo* showed that the changes were rapidly reversible. The rate of resynthesis after electrical stimulation was such that under these conditions the brain could synthesize approximately 7  $\mu$ g. acetylcholine per gm., or more than five times the normal brain content, in one minute. The changes were shown by the use of specially rapid methods and the results do not therefore disagree with those of other investigators who have used less rapid techniques.

These observations are clearly relevant to the problem of nervous transmission in the central nervous system. Various hypotheses of acetylcholine action have been discussed elsewhere and different aspects of the subject have been reviewed by Feldberg (9), Eccles (10), Nachmansohn (11) and Dale (12). It is known that the central synapses are highly sensitive to acetylcholine applied to the cerebral cortex, but there has been no direct evidence that acetylcholine is actively concerned in transmission in the central nervous system. The present work gives evidence that functional activity of the brain is associated with a fall in the total acetylcholine content. It gives no evidence of the mechanism involved, but it gives support to the view that nervous activity in the central nervous system involves the liberation and breakdown of acetylcholine.

Besides the changes in the acetylcholine level, stimulation has previously been shown to produce changes in the lactic acid and in other metabolites in the brain (1-3). The present observations on acetylcholine can be related to the other changes if it is assumed that the lactic acid formation corresponds to an increased utilization of carbohydrate required to supply energy for the resynthesis of acetylcholine during increased functional activity of the brain. Experiments *in vitro* suggest that the energy required for acetylcholine synthesis can be supplied through the mediation of the high-energy phosphate esters. In agreement with this view, the brain lactic acid level is low and the phosphocreatine is high during anesthesia, when acetylcholine synthesis may be expected to be minimal, while during electrical stimulation or convulsions the relations are reversed.

*Convulsions.* Acetylcholine produces spike discharges when applied after eserine to the cerebral cortex, and this has led to the suggestion that it may be specifically concerned in the mechanism of epileptic seizures (13). It was noted in the present experiments on electrically induced convulsions that the convulsions did not start until the acetylcholine had returned, after the initial fall, to the normal value (fig. 1). The convulsions also came to an end with the second fall in the acetylcholine level. This gave evidence that the two may be related and that an adequate acetylcholine level may be one of the requirements for convulsive activity of this kind. Activity would appear to be depressed if the acetylcholine falls to too low a value, as must happen if for any reason the rate of resynthesis cannot keep pace with the rate of acetylcholine liberation and breakdown. This agrees with the recent work of Hyde, Beckett and Gellhorn, who have shown that convulsive activity can be restarted, after it has come to an end, by the direct application of acetylcholine to the cerebral cortex (14). A similar explanation might be given to the increased frequency of seizures and of E.E.G. seizure patterns seen in epileptics during the earlier stages of anesthesia and in sleep, when the acetylcholine level may be expected to be high.

The observed changes in the acetylcholine level offer an explanation for some of the facts, but it is clear that other factors, apart from acetylcholine, would be needed to account for the occurrence of convulsions. The brain is apparently more irritable after electrical stimulation, for convulsions occur then when the acetylcholine level is no higher than normal. This abnormal irritability suggests the additional action of some other toxic factor and it may be relevant that ammonia, which is a powerful cerebral irritant, has been shown to be liberated in significant amounts by the action of convulsant drugs and electrical stimulation on the brain (3). The combined effects of acetylcholine and a toxic factor such as ammonia might offer a more satisfactory basis for interpreting the phenomena of convulsions.

#### SUMMARY

The acetylcholine content of the rat brain depends on the physiological state. It is increased in sodium pentobarbital anesthesia and in sleep; it is reduced in emotional excitement, in electrical stimulation and in convulsions. It would thus appear to vary inversely with the degree of activity of the brain. The changes are relatively large: the acetylcholine level in anesthesia is 300 per cent above that in convulsions. The fall in the brain acetylcholine in electrical stimulation is a transient and rapidly reversible effect. The rate of resynthesis of acetylcholine in the brain *in vivo* after electrical stimulation is of the order of 7  $\mu$ g. acetylcholine per gram per minute under these conditions.

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# INFLUENCE OF DESOXYCORTICOSTERONE ACETATE ON LIVER AND MUSCLE GLYCOGEN OF ADRENALECTOMIZED ANIMALS

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IT HAS often been stated that the corticosteroid hormones belong to two groups, one influencing carbohydrate metabolism, the other electrolyte metabolism. The representative of the first group is corticosterone; that of the second, desoxycorticosterone. It is often stated, especially in clinical literature, that only the 11-oxy-corticosteroids have an influence on carbohydrate metabolism (1-4). This has been contradicted by Verzár (5), who has claimed since 1940 that the main difference would not be one of quality but rather of velocity, desoxycorticosterone acting slower.

Grollman (6) writes: "Desoxycorticosterone fails to remedy the defect in carbohydrate metabolism observed in adrenal cortical insufficiency. . . ." And "Desoxycorticosterone and its 17-hydroxy-derivative fail to affect either the carbohydrate metabolism or the working capacity, whereas the corticosterone compounds exert. . . ." Ingle and Kuizenga (7) wrote in 1945, "11-desoxycorticosterone influences the carbohydrate metabolism of adrenalectomized animals in at least two different ways. First, . . . tends to restore normality to the circulatory mechanisms . . . ; second, . . . is weakly active in stimulating the formation of carbohydrate from non-carbohydrate sources. . . ."

Ingle (8) in his review of 1945 noted, "But it has been established beyond reasonable doubt that the compound 11-desoxycorticosterone is deficient in its ability to stimulate the formation of new carbohydrate from non-carbohydrate sources". It was said by Olson *et al.* (9) and often repeated by others that "There is now a wealth of evidence to indicate that neither those steroids which are not ketonic at C<sub>3</sub> and  $\alpha$ ,  $\beta$ -unsaturated in Ring A nor those which are without an oxygen substituent at C<sub>11</sub> have any influence upon carbohydrate and protein metabolism in either the intact or adrenalectomized animal."

In the present paper it is shown that desoxycorticosterone, if given in daily doses which keep adrenalectomized rats in a healthy state, has an action on glycogen formation and keeps normal values of liver and muscle glycogen. Nothing will be said about short-term experiments like those of Ingle (3) and Olson (4) *et al.*, where undoubtedly 11-desoxycorticosterones were inferior in their action, which will be analyzed later.

Three different types of feeding experiments on normal and adrenalectomized animals were used: *a*) carbohydrate rich, but protein and fat containing diet; *b*) forced feeding of glucose by stomach tube to starved animals; *c*) protein diet. Desoxycorticosterone acetate was given i.m. in oily solution.<sup>3</sup>

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## METHODS

Male rats of 70 to 160 gm. were adrenalectomized. Animals of the same age and weight were kept as controls. All were fed with the usual stock diet. Food was given twice daily at 9 A.M. and at 4 P.M. They were weighed before the afternoon feeding and part received i.m. injections of desoxycorticosterone acetate.

The animals were pithed and then bled from the carotids. One hundred to 300 mg. of liver and 300 to 500 mg. of trunk muscles were quickly weighed on a torsion balance and plotted in centrifuge tubes with 2 cc. boiling 20 per cent NaOH. Two and a half to 3 minutes for liver and 4 to 5 minutes for muscle elapsed from the death of the animals; this time was carefully kept.

The tissues were totally dissolved in the NaOH and then 3 cc. hot concentrated ethyl alcohol (96%) was added and mixed. Glycogen was precipitated in 30 minutes in the ice chest and then centrifuged. The precipitate was dissolved in 1 cc. hot 1 per cent NaCl, again precipitated with 2 cc. concentrated ethyl alcohol and again left for 30 minutes in the ice chest, centrifuged, washed with 2 cc. concentrated ethyl alcohol and then dried on a water-bath. The precipitate of glycogen was hydrolyzed with 4 cc. 2 N  $\text{H}_2\text{SO}_4$  in boiling water for  $1\frac{1}{2}$  hours, then made slightly alkaline with 5 cc. 2 N  $\text{Na}_2\text{CO}_3$ , transferred in 50 cc. bottles, filled with distilled water and in an aliquot part sugar estimated by Hagedorn-Jensen's method.

Glycogen is given in the tables as grams of glucose per 100 gm. wet tissue weight.

## EXPERIMENTAL RESULTS

*Animals on Mixed (Stock) Diet*

*Series I A and B.* In the first series, 17 adrenalectomized animals (series I A) on a mixed diet were injected with desoxycorticosterone. On the first 3 days 2 mg., later 1 mg., daily was given i.m. (The first injection was given just after adrenalectomy.) No injection was given on the day of estimation and the food was taken away  $1\frac{1}{2}$  hours before. The animals were killed in groups 10, 14, 16, and 20 days after adrenalectomy. With each group an approximately equal number, altogether 17 normal animals, was worked up for liver and muscle glycogen (series I B).

All the adrenalectomized animals were kept alive by desoxycorticosterone acetate. During the injections they increased in weight as did the other animals of the control series. To prove the action of desoxycorticosterone 5 animals of this series were not injected after the 20th day. Their weight curve immediately turned down and with constant loss of weight the animals died after 15 to 17 days. They were typically adynamic before death. Table 1 shows the glycogen content of liver and muscles of these animals (I A), compared with normals. There is no difference between these groups, either in liver or muscle glycogen.

*Series II A and B.* Fourteen adrenalectomized animals were daily injected with desoxycorticosterone. On the first day after operation they received 2 mg. and on the following days 1 mg. daily. A second group of 15 adrenalectomized animals was not treated with desoxycorticosterone. All these animals were killed in groups 2 to 5 days after adrenalectomy and their liver and muscle glycogen was estimated. Table 1 gives the mean values.

The mean values in table 1 are calculated from the values of the 2nd to 5th day,

from values of 11 untreated and 10 treated adrenalectomized animals. They show clearly the decrease of liver and muscle glycogen without treatment and the total normal values with treatment.

It is clear that after adrenalectomy in the untreated animals liver glycogen drops down, as is well known, in 1 to 5 days to very low values. In desoxycorticosterone-treated animals glycogen is mostly low on the first, but already normal on the 2nd and 5th day, since the first injection was given directly after the adrenalectomy. Desoxycorticosterone had little or no action on the first day of application but a complete action on glycogen later.

TABLE 1. MEAN VALUES OF LIVER AND MUSCLE GLYCOGEN IN NORMALLY FED ANIMALS

SERIES	GROUP	LIVER GLYCOGEN		MUSCLE GLYCOGEN	
		No. of Animals	Per cent	No. of Animals	Per Cent
I A	Adrenalectomized + DCA 10-20 days after adrenalectomy	15	$4.8 \pm 0.4$	17	$0.380 \pm 0.22$
I B	Normal controls	18	$4.8 \pm 0.3$	17	$0.415 \pm 0.27$
II A	Adrenalectomized, untreated, 2-5 days after adrenalectomy	11	$1.5 \pm 0.4$	11	$0.340 \pm 0.33$
II B	Adrenalectomized + DCA 2-5 days after adrenalectomy	10	$4.7 \pm 0.6$	10	$0.440 \pm 0.21$
Differences between		P for liver		P for muscle	
I B and II A		<0.01		0.05-0.1	
I B and II B		0.8-0.9		0.4-0.5	
I B and I A				0.3-0.4	
II A and II B		<0.01		0.01-0.02	

### Glucose Force-Fed Animals

In this and the next series the animals, which were kept on the mixed diet, were fasted for 24 hours and then were given by stomach tube 2 cc. 50 per cent glucose. Three hours later the animals were killed and glycogen estimated.

*Series III A and B.* Six normal animals were compared with 6 adrenalectomized animals on the 28th day after adrenalectomy after daily treatment with desoxycorticosterone in the same way as in the first series. Table 2 shows that in normal animals liver glycogen had recovered to 1.3 per cent, while in adrenalectomized animals it had reached 1.7 per cent. Muscle glycogen was 0.410 per cent in the normals and 0.475 per cent in the adrenalectomized. Thus no difference between the two groups was present.

*Series IV A and B.* Four adrenalectomized animals were treated from the first day after adrenalectomy with desoxycorticosterone acetate in the same way as above. The animals were fasted 24 hours, and then were given 2 cc. 50 per cent glucose by stomach tube 3 hours before used. They were killed on the 1st or 2nd day. Five more

adrenalectomized animals were untreated and killed together with the former. The result is shown in table 2. Desoxycorticosterone restored the ability to form liver glycogen in the adrenalectomized animals by the first day after adrenalectomy. Muscle glycogen seemed not quite restored on the second day.

### *Protein-Fed Animals*

The possibility has often been suggested that the adrenal cortical hormones influence glucose formation from proteins. In the following experiments we compared normal and adrenalectomized animals on a protein diet, which was prepared in the following way: Lean beef was cooked in water, finely minced through a meat grinder and cooked once again. Twenty gm. was fed daily to each rat but was not totally

TABLE 2. MEAN VALUES OF LIVER AND MUSCLE GLYCOGEN IN 24-HOUR STARVED, GLUCOSE FORCE-FED ANIMALS

SERIES	GROUP	LIVER GLYCOGEN		MUSCLE GLYCOGEN	
		No. of Animals	Per cent	No. of Animals	Per Cent
III A	Normal controls	6	$1.3 \pm 0.18$	6	$0.410 \pm 0.40$
III B	Adrenalectomized + DCA 28 days after adrenalectomy	6	$1.7 \pm 0.18$	6	$0.475 \pm 0.25$
IV A	Adrenalectomized + DCA 1-2 days after adrenalectomy	4	$1.8 \pm 0.23$	4	$0.380 \pm 0.08$
IV B	Adrenalectomized, untreated, 1-2 days after adrenalectomy	5	$0.4 \pm 0.22$	5	$0.280 \pm 0.07$
Differences between		P for liver		P for muscle	
III A and III B		0.1-0.2		0.1-0.2	
IV B and IV A		<0.01		0.7-0.8	

consumed. Vitamins B<sub>1</sub> and B<sub>2</sub> and A and D in the form of cod liver oil were added to the daily dose.

Groups of normal and adrenalectomized animals were killed on the same days, and estimations of glycogen in liver and muscle were made.

*Series V A.* Seven normal rats (of 150-200 gm.) were fed with the meat diet 20 to 24 days. All had decreased in weight from the beginning of the pure meat feeding by 19 to 41 gm., but were in good health. They showed very poor or no fat reserves. They were killed 20, 22 and 24 days after the feeding began. Their livers had a glycogen content of 3.543 gm. per cent, their muscles 0.514 gm. per cent.

*Series V B.* Fourteen rats were kept for one week on pure meat diet and then adrenalectomized. They were not treated with hormone and were killed on the 3rd to 9th day after adrenalectomy, when their body weight had declined from the day of adrenalectomy by 4 to 69 gm. and adynamia was present. The liver glycogen was

extremely low. The mean value was 0.081 gm. per cent. Muscle glycogen had also much decreased to 0.140 gm. per cent mean value.

*Series V C.* Twenty-three adrenalectomized animals, treated i.m. with 2 mg. desoxycorticosterone acetate daily, were used between the 3rd and 13th day after adrenalectomy. Fifteen animals were fed on a pure meat diet 3, 7 and 10 days before the adrenalectomy and then again after it. In 8 animals meat was fed 14 days before the adrenalectomy, and from then on. All animals were treated with 2 mg. desoxycorticosterone daily. The glycogen content of the liver showed a mean value of 0.890 gm. per cent. In 10 of these 23 animals the value was above 1.1 gm. per cent. In 9 cases, however, it was under 0.4 gm. per cent, but three times under 0.2 gm. per cent, as in untreated animals. Their muscle glycogen was 0.480 gm. per cent mean value.

There is no doubt that the majority of desoxycorticosterone-treated animals could produce glycogen from protein since their liver glycogen was much higher than that of untreated animals. In half of the cases it reached about half the value of normals.

*Series VI A and VI B.* Since, however, no explanation could be given for the low values in about half of the former series, more experiments were done on these lines. The desoxycorticosterone-treated animals had lost considerable weight in this last series. It was thought that they were not in an optimal state of compensation with 2 mg. desoxycorticosterone daily. Further experiments were being made therefore with 3 mg. desoxycorticosterone daily. In this series the adrenalectomized animals did not lose weight.

Eight normal controls (*series VI A*) were always taken together with the adrenalectomized animals, so that they were for the same time interval on the meat diet. Thirteen adrenalectomized animals (*Series VI B*) at the time of adrenalectomy had been on the meat diet for 6 days. They were killed on the 5th (2), 6th (3), 9th (4) and 10th (4) day after adrenalectomy. The normal controls had a liver glycogen mean value of 3.575 per cent and a muscle glycogen of 0.848 per cent. Animals treated daily with 3 mg. DCA had a liver glycogen mean value of 2.277 per cent with muscle glycogen 0.820 per cent. Table 3 shows all the results of the protein feeding experiments.

From these results it seems to be quite certain that desoxycorticosterone-treated animals on 3 mg/day can produce glycogen from protein. It is questionable whether the difference of the glycogen content of the liver between the normal controls of *series VI A* and desoxycorticosterone-treated adrenalectomized animals (*series VI B*), 3.5 per cent and 2.3 per cent respectively, means an insufficient activity of 3 mg. desoxycorticosterone as in animals with 2 mg/day desoxycorticosterone. However muscle glycogen shows no such differences.

It is certain that 2 mg. desoxycorticosterone daily did not replace the adrenal function completely. While glycogen was produced from protein, this was certainly possible only in a much smaller degree. This is a striking difference to the glycogen production on a carbohydrate diet or glucose-forced feeding. Three mg/day however was sufficient for muscle glycogen, and nearly sufficient for liver glycogen production to become normal.

## DISCUSSION

Three different types of experimental procedure were used. In the first we kept normal and adrenalectomized rats on a mixed diet, the usual stock diet. Normal adrenalectomized untreated and desoxycorticosterone-treated rats were compared. Liver and muscle glycogen was analyzed when the latter were kept alive for various lengths of time. There was no difference between the liver and muscle glycogen of normal and desoxycorticosterone-treated adrenalectomized rats.

In another type of experiment the rats were kept on the same stock diet, were fasted for 24 hours and then force-fed with 1 gm. glucose; 3 hours later glycogen

TABLE 3. MEAN VALUES OF LIVER AND MUSCLE GLYCOGEN IN PURE PROTEIN-FED ANIMALS

SERIES	GROUP	LIVER GLYCOGEN		MUSCLE GLYCOGEN	
		No. of Animals	Per Cent	No. of Animals	Per Cent
V A	Normal controls	7	$3.543 \pm 0.46$	7	$0.514 \pm 0.03$
V B	Adrenalectomized untreated	14	$0.081 \pm 0.016$	14	$0.140 \pm 0.018$
V C	Adrenalectomized treated with 2 mg. DCA	23	$0.890 \pm 0.155$	23	$0.480 \pm 0.033$
VI A	Normal controls	8	$3.575 \pm 0.40$	8	$0.848 \pm 0.060$
VI B	Adrenalectomized treated with 3 mg. DCA	13	$2.277 \pm 0.18$	13	$0.820 \pm 0.033$
Differences between		P for liver		P for muscle	
V A and V B		<0.01		<0.01	
V A and V C		<0.01		>0.9	
V B and V C		<0.01		<0.01	
VI A and VI B		<0.01		0.6-0.7	

was estimated. Again there was no difference between normal and adrenalectomized, desoxycorticosterone-treated animals, while untreated animals had very low values.

The third type of experiment was done on animals on a meat (i.e., practically pure protein) diet. It was noticed that in these experiments 3 mg. of desoxycorticosterone had to be given daily to keep the animals in such a normal state that liver glycogen was nearly and muscle glycogen quite equal to that of the normals. It seems possible that the usual doses of desoxycorticosterone to keep adrenalectomized animals alive on a stock diet are not equal to those which are needed for animals on a pure meat (protein) diet. There seems to be reason to believe that especially high hormone doses are needed for the latter.

Thus desoxycorticosterone restores glycogen production to normal from carbohydrate or protein in liver and muscles of adrenalectomized animals which are kept alive for some time.

All of these experiments were made on animals which were treated for 2 to 28 days with desoxycorticosterone. Nothing is said about the possibility of influencing glycogen production in a shorter period. The reason for these experiments was to show that desoxycorticosterone has a strong glycogenetic action if given continually. It brings liver and muscle glycogen to normal.

#### SUMMARY

Desoxycorticosterone-treated rats are able to restore liver and muscle glycogen after several days to normal values. This was found to be true with rats on a mixed diet, or starved and then glucose force-fed, or on a protein diet.

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# COMPARISON BETWEEN GLYCOGENETIC PROPERTY OF DESOXYCORTICOSTERONE, 11-DEHYDRO-17-HYDROXY-CORTICOSTERONE (COMPOUND E) AND ADRENAL CORTICAL EXTRACT

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IN THE preceding paper (1) it was shown that desoxycorticosterone acetate (DCA) has a glycogenetic effect in animals, which are kept alive with this cortical steroid. This paper demonstrates the difference between this formerly so called 'salthormone' and a typical 'carbohydrate hormone,' 11-dehydro-17-hydroxycorticosterone (Compound E), which has recently been available to us in large enough quantities.<sup>2</sup> Analytical methods were the same as in the former paper (1). All animals were kept on the pure protein diet described there, consisting of cooked meat with the necessary vitamins added. Male rats of 130 to 180 gm. were used.

## EXPERIMENTS

*Experiments with Compound E and DCA. Series I* was done on 33 rats (table 1). The animals were adrenalectomized after 13 to 14 days on the protein diet. Adrenalectomized animals, if they survived 4 to 7 days without treatment, had extremely low liver and muscle glycogen values. If they were treated for 7 to 14 days after the adrenalectomy with 2 mg. DCA or 2 mg. Compound E daily, when all animals were healthy and lively, liver glycogen was 2.26 gm. per cent in the DCA and 3.57 gm. per cent in the E group. There was no difference in muscle glycogen.

Normal animals were killed together with the former. They were on the protein diet the same length of time. The liver glycogen was 1.97 gm. per cent and the muscle glycogen 0.49 gm. per cent.

In *series II* we used 25 animals (table 2). They were kept on the protein diet 3 days before adrenalectomy. The normal animals were kept as long on this diet as the adrenalectomized. They had a liver glycogen value of 1.9 gm. per cent.

After adrenalectomy and a daily treatment of 4 mg. DCA after 5 to 16 days, we found 1.2 gm. per cent liver glycogen. However, only 1 mg./day of Compound E gave 1.9 gm. per cent liver glycogen.

All our data on animals on protein diet after prolonged treatment have been collected in table 3.

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<sup>2</sup> We wish to thank Ciba A. G., Basle, for the desoxycorticosterone-acetate; Merck & Co., Rahway, for Compound E and Upjohn Co., Kalamazoo, for the adrenal cortical extract, for these experiments.

From these experiments on 77 adrenalectomized animals kept alive for 5 to 16 days after the adrenalectomy with corticosteroids, it seems that while DCA can produce normal liver and muscle glycogen values in doses of 2 to 3 mg/day, Compound E is somewhat superior to it. Thus in *series I* 2 mg/day DCA produced 2.2 and Compound E 3.6 per cent liver glycogen. In *series II* 1 mg. Compound E daily produced more liver glycogen than 4 mg. DCA (but 4 mg. DCA did not produce more than 2 mg. DCA in the other series). Muscle glycogen showed no difference.

TABLE 1. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

DAYS AFTER ADRENAL- ECTOMY	'E' TREATED, 2 MG/DAY		DCA TREATED, 2 MG/DAY		UNTREATED		NORMAL CONTROLS	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
4					0.10	0.16		
5					0.10	0.18		
6					0.10	0.15		
7	1.90	0.50	3.90	0.77	0.10	0.15	3.00	0.59
	1.60	0.65	4.30	0.65	0.10	0.12		
9	6.80	0.85	1.10	0.66			1.10	.55
	3.40	0.68	0.75	0.45				
	4.20	0.60						
11	3.50	0.36	0.30	0.21			1.90	0.26
	3.50	0.48	1.30	0.36				
	4.10	0.44						
12	3.50	0.59	4.30	0.79			3.00	0.45
	3.70	0.54	1.70	0.67				
13			2.30	0.60				
14	3.10	0.59	2.60	0.61			1.90	0.58
							1.10	0.48
							1.80	0.54
Mean values	3.57±0.16	0.57±0.04	2.26±0.47	0.58±0.06	0.10 <sup>1</sup>	0.15±0.00	1.97±0.30	0.49±0.26
No. of animals	11		10		5		7	

<sup>1</sup> ±0.05 as limit of error of estimation.

*Experiments with Adrenal Cortical Extract (Upjohn).* In *series III*, 10 protein-fed animals were treated after adrenalectomy with 0.5 cc. extract injected daily i.m. Three animals died. Seven surviving animals showed between the 7th and 11th day the following very low mean values: liver glycogen 0.15 gm. per cent, muscle glycogen 0.26 gm. per cent.

In *series IV*, 6 protein-fed animals were adrenalectomized after 4 days on the diet. They were then treated daily with 2 cc. extract i.m. for 10 days. The extract was injected once daily. Glycogen analyses were made 24 hours after the last injection. Parallel animals were untreated, or treated with 2 mg. DCA/day. The results are shown in table 4.

TABLE 2. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

DAYS AFTER ADRENALECTOMY	'E' TREATED, 1 MG/DAY		DCA TREATED, 4 MG/DAY		NORMAL CONTROLS	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
5	1.40	0.58	0.44	0.41	2.20	0.66
	1.20	0.37	0.30	0.40		
7	2.70	0.43	1.00	0.55	1.60	0.50
	3.10	0.41	2.70	0.47		
13	2.90	0.63	0.20	0.50	0.79	0.52
	0.97	0.43	0.10	0.23		
14	1.00	0.56	0.55	0.82	2.30	0.58
	0.73	0.64	1.50	0.80		
16	2.50	0.38	2.80	0.71	2.70	0.41
	2.10	0.49	2.30	0.70		
Mean values	$1.90 \pm 0.28$	$0.49 \pm 0.03$	$1.20 \pm 0.33$	$0.56 \pm 0.06$	$1.90 \pm 0.34$	$0.53 \pm 0.04$
No. of animals	10		10		5	

TABLE 3. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

SERIES	TREATMENT	DAYS	GLYCOGEN CONTENT		NO. OF ANIMALS
			Liver	Muscle	
V C <sup>1</sup>	DCA	maxim.	gm. %		
	2 mg/day	13	$0.89 \pm 0.155$	$0.48 \pm 0.033$	23
I	2	14	$2.26 \pm 0.47$	$0.58 \pm 0.06$	10
VI B <sup>1</sup>	3	13	$2.28 \pm 0.018$	$0.82 \pm 0.033$	13
II	4	16	$1.20 \pm 0.33$	$0.56 \pm 0.06$	10
II	E 1	16	$1.90 \pm 0.28$	$0.49 \pm 0.03$	10
I	2	14	$3.57 \pm 0.16$	$0.57 \pm 0.04$	11

<sup>1</sup> See the former paper (1).

TABLE 4. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

DAYS AFTER ADRENALECTOMY	TREATMENT	GLYCOGEN CONTENT	
		Liver	Muscle
		gm. %	
10	Untreated	0.15	0.49
10	Untreated	0.10	0.20
10	2 cc. untreated extract	0.18	0.63
10	2 cc. untreated extract	0.16	0.56
10	2 mg. DCA	1.30	0.76
10	2 mg. DCA	1.30	0.78

Thus we were unable to keep the glycogen content of the liver even fairly normal with 0.5, or 2.0 cc. extract/day, while in parallel experiments DCA gave the usual normal (or nearly normal) glycogen values in the liver. In muscles 2 cc. of the extract gave relatively high glycogen values, which, however, still were lower than with desoxycorticosterone.

*Short-time Experiments.* Since it is known from the experiments of Reinecke and Kendall (2), Olsen (3) and Ingle (4) that in experiments of a few hours' duration the

TABLE 5. GLYCOGEN CONTENT OF LIVER AND MUSCLE, PER CENT WET WEIGHT

HOURS AFTER INJECTION	DCA 2 MG. GLYCOGEN CONTENT		'E' 2 MG. GLYCOGEN CONTENT		EXTRACT 3 CC. GLYCOGEN CONTENT	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
	gm. %		gm. %		gm. %	
6	0.68	0.56	1.80	0.86	0.14	0.37
	0.10	0.48	1.70	0.64	0.11	0.25
	0.10	0.36				
	0.20	0.38				
	0.12	0.41				
12	0.80	0.43	0.74	0.52	1.90	0.39
	1.40	0.75	3.60	0.85	0.66	0.23
	1.50	0.41	4.90	0.75		
	0.84	0.25				
24	0.75	0.95	4.10	0.87	0.20	0.32
	0.20	0.41	4.50	0.87	0.34	0.37
	0.41	0.38	6.00	0.83		
	0.50	0.65	1.90	0.55		
	0.64	0.43				
	1.60	0.59				
	1.20	0.66				
48	0.10	0.41	0.55	0.60		
	0.12	0.66	1.10	0.64		
72	0.10	0.39	0.30	0.45		
	0.30	0.46	0.50	0.47		
			0.52	0.43		
			2.00	0.39		

so-called 'carbohydrate hormones' show much higher glycogenetic capacity, we made experiments of much shorter duration.

The animals were fed one week on the protein diet, then adrenalectomized and kept alive with daily injections of 2 mg/day DCA, during 6 days. On the 7th day they did not receive DCA. Before they were fed on the 8th day at 9 A.M. they were injected with either DCA (2 mg.), Compound E (2 mg.) or cortical extract (3 cc.). Some animals were killed after 6 hours, other at 12, 24, 48, or 72 hours later. The results of 41 experiments are shown in table 5.

## DISCUSSION

From the above it can be confirmed that Compound E (11-dehydro-17-hydroxy-corticosterone) has a definitely stronger action on carbohydrate metabolism in protein-fed animals than desoxycorticosterone in the short experiments. Desoxycorticosterone had no effect after 6 hours, while Compound E by this time gave high liver and muscle glycogen values. After 12 to 24 hours with DCA, 1.2 to 1.6 gm. per cent liver glycogen was reached, while with Compound E values more than three times as high (3.6-6.0 gm. per cent glycogen) were seen. After 48 hours the DCA

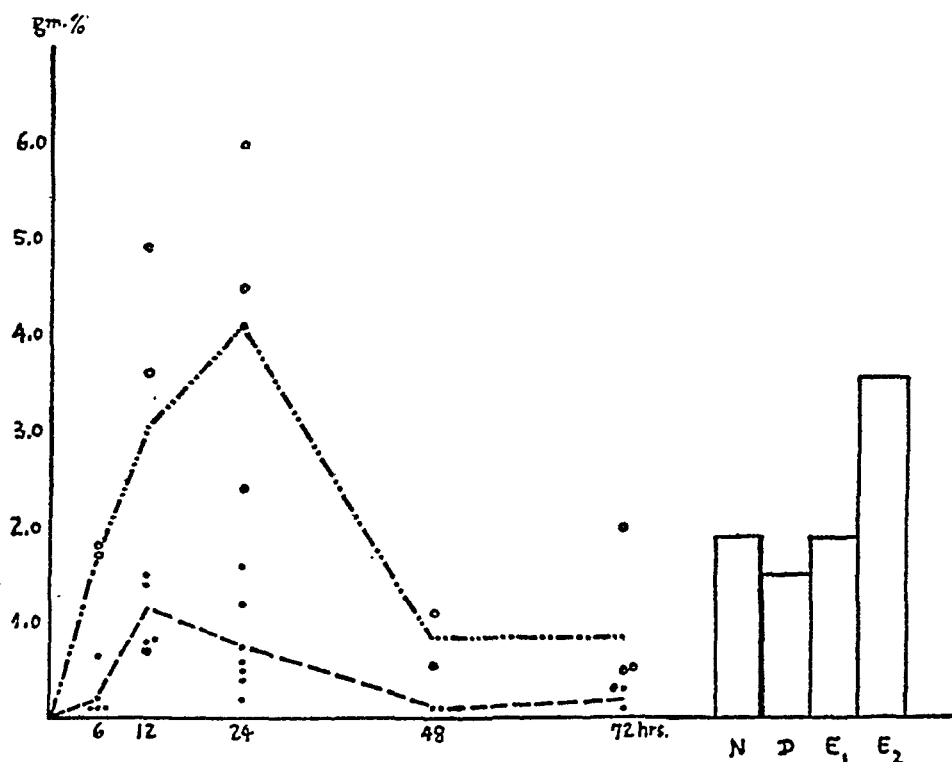


Fig. 1. LIVER-GLYCOGEN after single injection and after continuous treatment with DCA or Compound E. In the left part the liver glycogen content at the intervals of 6, 12, 24, 48 and 72 hours after a single injection of Compound E or DCA is plotted (table 5). In the right part the columns give liver glycogen middle values of DCA and E in long-term experiments of 3 to 16 days' duration from all experiments of table 3 and normal controls from table 1 and 2 together. — — — DCA 2 mg. single injection. — · — · —, E 2 mg. single injection. N, normal controls. D, DCA 2 to 4 mg/day during 3 to 16 days; E<sub>1</sub>, Compound E 1 mg/day during 3 to 16 days. E<sub>2</sub>, Compound E 2 mg/day during 3 to 16 days.

effect had totally disappeared, but with Compound E some small activity was still recognizable.

The difference between DCA and Compound E was, however, smaller in experiments with daily treatment over a longer period of 7 to 15 days. Then desoxycorticosterone-treated animals also showed high values of liver and muscle glycogen. The number of animals which we treated was not large enough to give a more quantitative comparison in such experiments of long duration. However, a relation of 1 to 2 in favor of Compound E is probable. The mean value of the DCA-treated animals reached the glycogen content of normal protein-fed rats.

Thus the action of Compound E is especially quick and strong compared with DCA if used in single doses. It is much less different in chronic experiments. It has been supposed for several years (5, 6) that the difference between the action of the two groups of steroids with regard to the carbohydrate metabolism is not qualitative but mainly quantitative. Compound E has a quick and strong action on glycogen formation. DCA acts much slower and therefore less after a single injection. But it brings glycogen production to about normal values if given in daily repeated doses (see fig. 1). We have not studied the relation of these two steroids in animals on a carbohydrate diet as yet.<sup>3</sup>

Adrenal cortical extract in quantities of 2 cc/day, injected intramuscularly, showed in chronic experiments of 10 days' duration and short-time experiments of 6, 12 or 24 hours' duration, less activity than 2 mg. DCA. We had not enough material to test greater doses.

It is shown, however, in the former and present papers, that especially in long-term experiments the difference in glycogen production between a so-called 'electrolyte hormone' (as DCA) and a 'carbohydrate hormone' (as Compound E) is not a qualitative one, but a quantitative one only.

#### SUMMARY

The action of 11-dehydro-17-hydroxycorticosterone (Compound E), desoxycorticosterone (DCA) and adrenal cortical extract upon glycogen formation in liver and muscle of adrenalectomized rats on a pure protein diet has been tested. Compound E has a much stronger and more rapid effect than DCA in short-time experiments. The difference is smaller in experiments with long periods of treatment, where desoxycorticosterone also brings the liver glycogen values of adrenalectomized rats to near normal.

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# INFLUENCE OF ESTROGENS ON THE ACUTE X-IRRADIATION SYNDROME

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IT HAS been reported that estradiol benzoate, administered in a single dose 9 to 10 days prior to total-body x-irradiation, increases the resistance of male mice to the x-rays (1-3). On the other hand, injection of the estrogen on the day of irradiation appears to potentiate toxicity. The mechanism whereby pretreatment with an estrogen can alter the toxic effects of x-rays is unknown. Although many phases of the biologic response to ionizing radiations have been well defined experimentally, the factors contributing to radiosensitivity and the basis of radiation death still remain largely a matter for conjecture. It seemed important, therefore, to investigate further this interesting phenomenon of estrogen protection against x-rays.

## METHODS

Male and female CF<sub>1</sub> mice, weighing 20 to 25 grams and maintained on a diet of Derwood Checkers and water *ad libitum*, were used in these studies. Animals of the same sex were used in individual experiments. For the irradiation, mice were placed in individual cellulose acetate exposure cells and were given a total-body exposure in groups of 16. Equal numbers of controls and experimentals were irradiated in each exposure. The radiation factors were 200 kv., 15 ma., 0.5 mm. Cu and 3.0 mm. Bakelite filters, target distance 10.8 cm., dose rate 20 r per minute. Male mice received 500 r, females 550 r (measured in air). Several types of experiments were performed. These are considered under the following headings.

*Influence of Time of Injection of Estradiol Benzoate on Survival after X-Irradiation.* Estradiol benzoate (0.166 mg. in 0.1 cc. sesame oil per mouse I.M.) was given in a single injection at either 35, 25, 15, 10, 5, or 2 days before irradiation, or at the time of exposure or 2 days afterward. Irradiated controls for the above groups received injections of sesame oil (0.1 cc. I.M.) at comparable times.

*Effect of Estradiol Benzoate on Peripheral Blood Count and on Organ Weights.* Blood sampling was accomplished by making a deep cut in the tail with a sharp razor blade and using a free flow of blood. Determinations on the peripheral blood included: total leucocyte count, erythrocyte count, hemoglobin and a differential leucocyte count made on dry smears prepared with Wright's stain. After the blood sample was obtained, the mice were weighed and killed with sodium pentobarbital. The adrenals, spleen, thymus, inguinal lymph nodes and kidneys were removed, dissected free of fat and weighed on a torsion balance. Certain tissues were then prepared for microscopic examination. The histologic observations have not been completed. Animals in the different groups which were to be bled and killed at various intervals were distributed in random fashion among the different cages and levels of the mouse rack.

It was felt that this sampling procedure for obtaining the hematologic data would be superior to the frequent repeated bleeding of mice which would otherwise be necessary. Irradiated and nonirradiated mice pretreated with a single injection of estradiol benzoate were compared with similar mice receiving sesame oil.

*Importance of Adrenals for Protective Action of Estradiol Benzoate.* Mice were bilaterally adrenalectomized under sodium pentobarbital anesthesia 3 to 7 days prior to injection of estradiol benzoate or sesame oil. Part of the animals were irradiated 10 days after injection of the latter materials, the remainder served as nonirradiated controls. All of the adrenalectomized mice were given 1 per cent sodium chloride in the drinking water. In addition, some of the mice received daily subcutaneous injections of 0.2 cc. aqueous adrenal cortical extract (Wilson).

TABLE 1. EFFECT UPON SURVIVAL RATES OF ALTERING THE TIME OF INJECTION OF ALPHA-ESTRADIOL BENZOATE (0.166 MG/MOUSE I.M.) RELATIVE TO X-IRRADIATION (500 R) OF C57 MALE MICE

TIME OF INJECTION RELATIVE TO X- IRRADIATION, DAYS	ESTRADIOL					SESAME OIL				
	% survival, weeks after irradiation					% survival, weeks after irradiation				
	Total no. of mice	1	2	3	4 <sup>1</sup>	Total no. of mice	1	2	3	4
2	32	78	9	0	0					
0	54	83	20	15	13	39	95	46	31	28
-2	32	97	36	22	22					
-5	54	94	67	63	61	18	95	61	33	33
-10	126	88	80	80	79	211	91	39	28	28
-15	29	93	81	69	69	30	77	47	44	44
-25	26	85	45	42	42	25	96	32	32	32
-35	21	85	28	19	19	29	89	14	7	7

<sup>1</sup> Bold face figures are significantly different ( $P < .05$ ) from the 4-week mean survival of the combined sesame oil controls (28% of 352 mice).

*Specificity of the Estrogen Response.* Benzestrol (0.5-1 mg/mouse I.M.), progesterone (0.1-0.2 mg. I.M.) alone or in combination with estradiol benzoate, and testosterone propionate (0.1-1.0 mg. I.M.) were given to mice 10 days before x-irradiation. Survival rates were determined as a measure of sensitivity to the radiation. Estradiol benzoate was also administered to mice 10 days before injection of a nitrogen mustard—methyl (2, 2'-dichloro) diethyl amine HCl, 3.5  $\mu$ g/gm. subcutaneously—and toxicity compared with that of untreated controls.

## RESULTS

*Time of Injection of Estradiol Benzoate.* Estradiol benzoate exerts its maximal protective influence when it is given 10 days before x-irradiation (28-day survival, 79% estrogen treated; 28% control irradiated,  $P < .001$ ). Estrogen injection at 5 and 15 days before exposure is also effective while pretreatment at 2, 25 or 35 days is apparently without influence on radiosensitivity. When estradiol is administered at the time of irradiation or 2 days after the exposure, toxicity to x-rays appears to

be enhanced, both in terms of absolute survival and of survival time. These findings are summarized in table 1.

*Peripheral Blood Changes.* The hematologic picture, following the single injection of estradiol benzoate, is characterized by a decrease in both the heterophils<sup>1</sup> and lymphocytes (figs. 1-3). Maximal depression occurs around 10 to 14 days after the injection. The heterophil change ( $p < .05$ ) is more marked and appears to be somewhat more consistent than the lymphocyte response which is of borderline statistical significance. The erythrocyte count is apparently not influenced appreciably by the estrogen (fig. 4).

Although the total leucocyte count is decreased to the same level in both the estrogen pretreated and sesame oil control groups after their x-irradiation, there is a

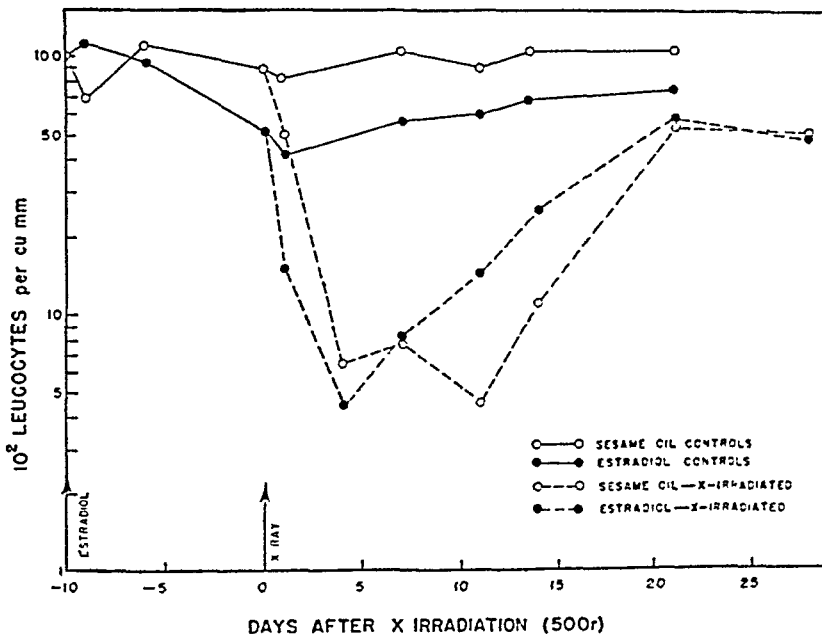


Fig. 1. EFFECT ON TOTAL LEUCOCYTE COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF<sub>1</sub> male mice.

rather consistent difference in the time course of the response, maximal depression and recovery occurring earlier in the former (fig. 1). The more rapid recovery of leucocytes in the irradiated mice which received a prior injection of estrogen appears to reside in the heterophil component (figs. 2 and 3). This difference in heterophil recovery in the estrogen and control irradiated groups is significant at the 5 per cent level. Erythrocytes in the estrogen pretreated irradiated group are decreased by about 25 per cent 7 days after the exposure but normal values are seen at 11 and 14 days when the red count of the irradiated controls is reduced by 45 per cent (fig. 4). Similar changes are seen in blood hemoglobin.

*Organ Weights.* Estradiol has no appreciable effect on the weight of the spleen, inguinal nodes or kidney but does increase adrenal weight and accelerate thymic in-

<sup>1</sup> The term heterophil is used to designate the polymorphonuclear leucocyte homologous to the neutrophil in human blood. In the mouse the heterophil is stained with acid dyes.

volution (figs. 5 and 6). Adrenal weight is elevated by 35 to 40 per cent 4 days after estrogen administration and then slowly declines, reaching the control level around 35 days after injection. Radiation-induced involution of the spleen, thymus, and inguinal nodes is not altered by pretreatment with estradiol. However, a somewhat greater increase in adrenal weight is seen in the estrogen-treated irradiated mice.

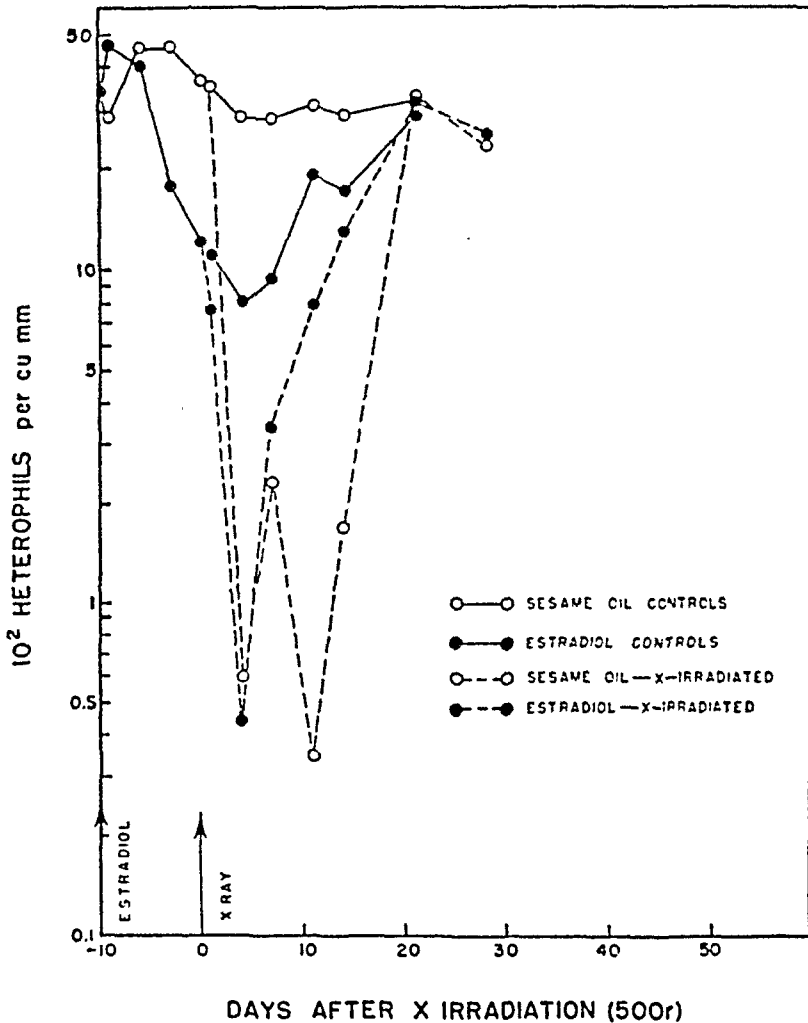


Fig. 2. EFFECT ON HETEROPHIL COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF<sub>1</sub> male mice.

*Importance of Adrenals.* Adrenalectomized mice given 1 per cent sodium chloride in the drinking water show a decreased survival time after x-irradiation, although final toxicity remains unchanged. When such operated mice receive a daily adrenal cortical supplement in addition to sodium chloride, they exhibit a sensitivity to radiation which is identical with that of the unoperated controls. Estradiol benzoate administered to adrenalectomized mice maintained only with sodium chloride exerts some protective influence, but this is considerably less than that observed in the operated animals receiving an adrenal cortical supplement (table 2). Estradiol protection in the latter is equivalent to that seen in mice with adrenals (fig. 7).

*Specificity of Estrogen Response.* Preliminary experiments reveal that benzenestrol, a synthetic estrogen, is effective in protecting male and female mice against x-irradiation (table 3). Progesterone and testosterone, on the other hand, appear to be in-

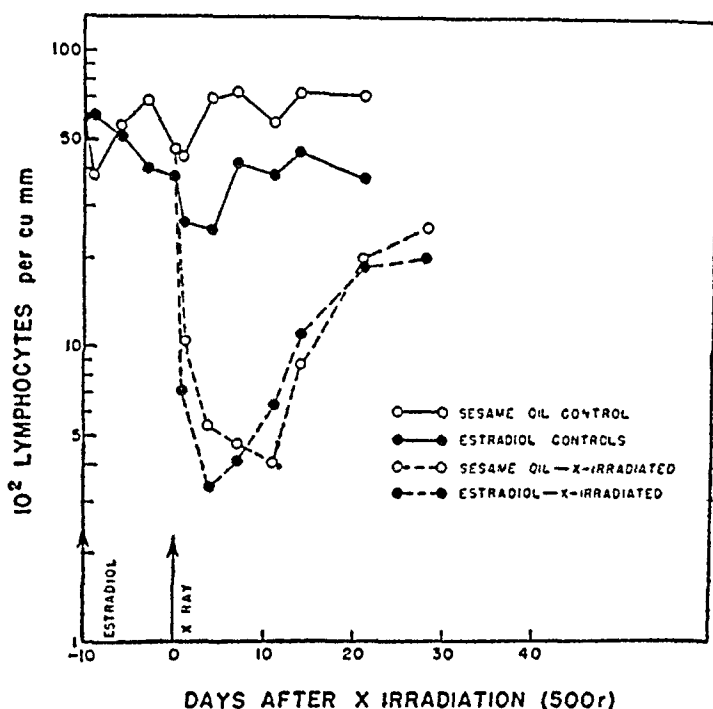


Fig. 3. EFFECT ON LYMPHOCYTE COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF<sub>1</sub> male mice.

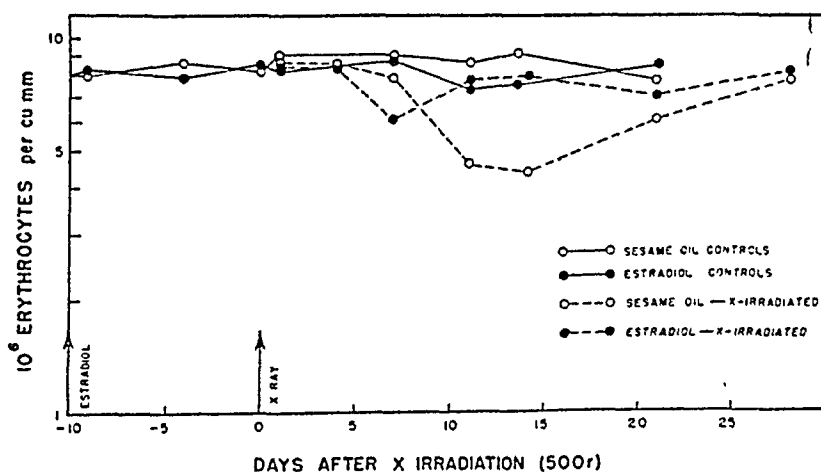


Fig. 4. EFFECT ON ERYTHROCYTE COUNT of a single injection of estrogen 10 days before x-irradiation with 500 r. Each point represents the average count of 10 to 14 CF<sub>1</sub> male mice.

effectual. Combining progesterone with estradiol does not alter the ameliorating influence of the estrogen. These observations are summarized in table 4. Administration of estradiol to mice 10 days before poisoning with a nitrogen mustard does not influence toxicity (table 5).

## DISCUSSION

Our findings confirm the earlier observation of Treadwell, Gardner and Lawrence (1) that pretreatment with estradiol benzoate improves the survival of male mice after lethal x-irradiation. It has been demonstrated further that the protective effect of estrogens occurs in female mice as well and that Benzestrol, a synthetic estrogen, but not progesterone and testosterone, is also effective. The time of injection of estro-

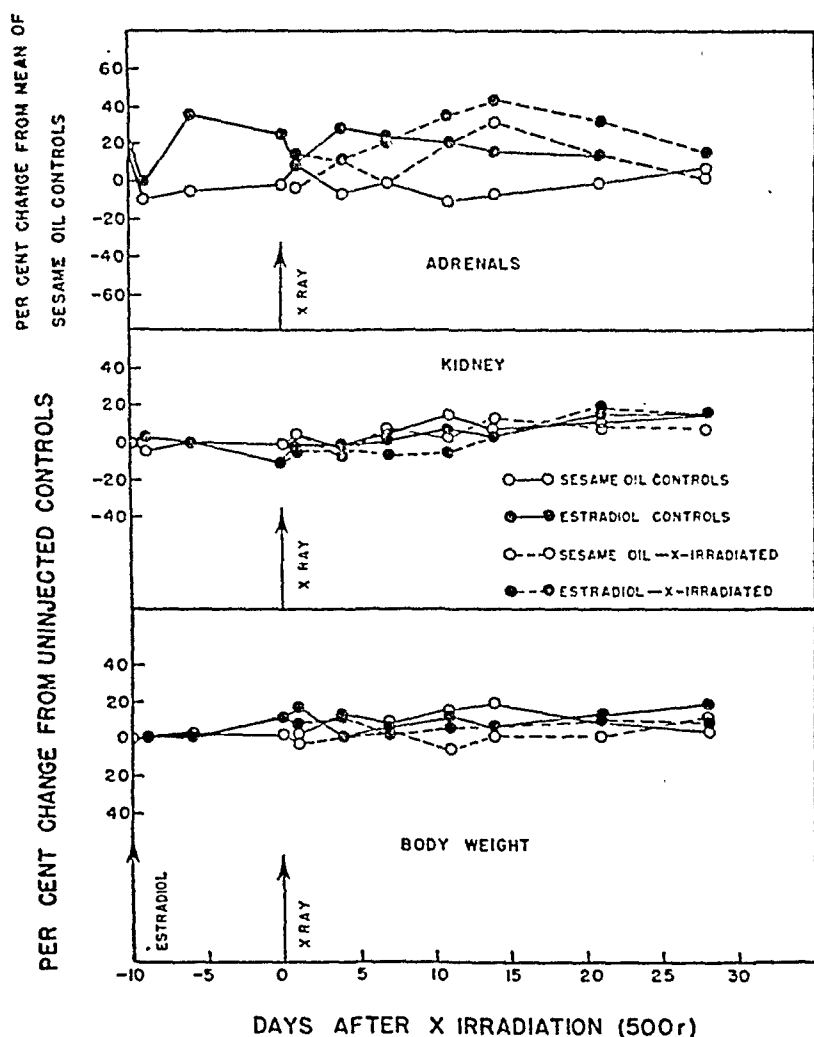


Fig. 5. EFFECT ON ORGAN WEIGHTS of estrogen administration 10 days before x-irradiation with 500 r. Each point represents the average of values obtained on 5 to 7 CF<sub>1</sub> male mice.

gen relative to the time of irradiation appears to be critical, for estradiol is most effective when it is given 10 days before the exposure. Estrogen injection 5 or 15 days before irradiation is also protective but administration at other intervals fails to increase survival and, indeed, may even potentiate toxicity. Although x-irradiation and nitrogen mustard intoxication are rather similar in many respects, estradiol, in dosage sufficient to protect against x-rays, does not influence survival after poisoning with a nitrogen mustard.

Since it is known that thymic involution and adrenal enlargement may occur

after estrogen administration (4, 5), the possibility was first considered that estradiol, perhaps through the intermediary of the adrenal cortex, rendered lymphoid tissue more resistant to irradiation. However, the hematologic data which we have obtained reveal that the lymphocyte count, although depressed by the estrogen, responds to irradiation identically in the estrogen-treated and control-irradiated animals. More-

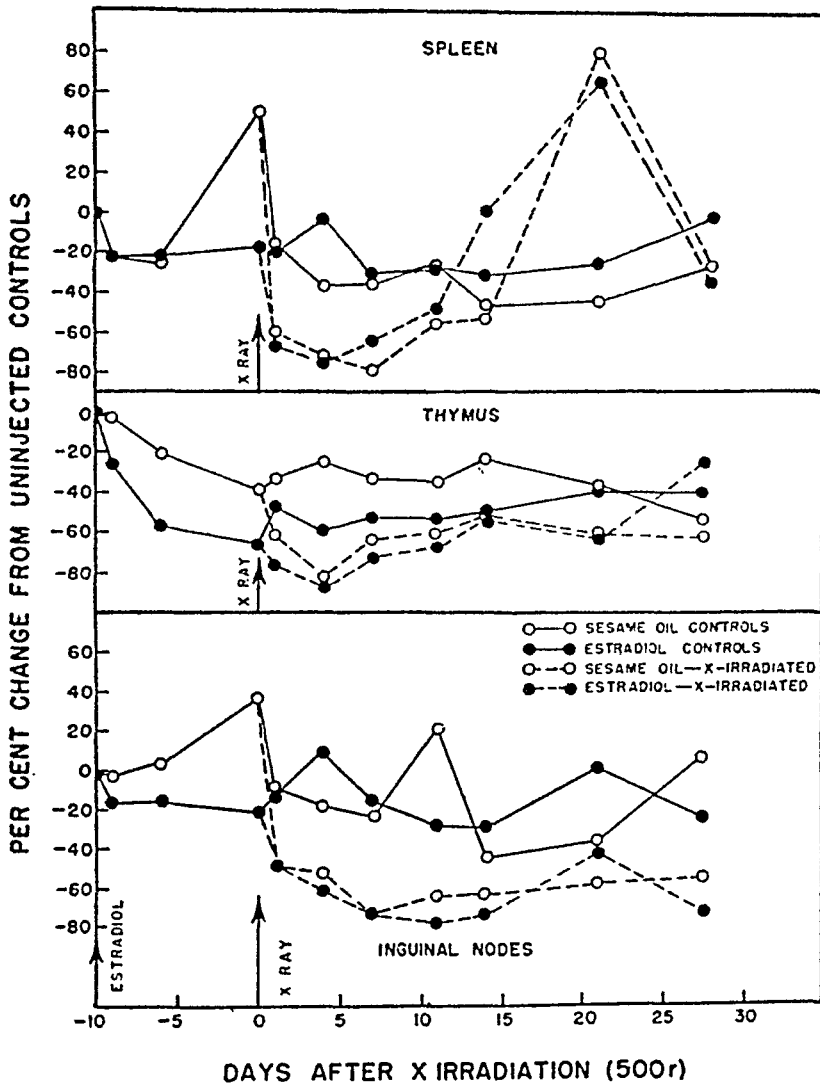


Fig. 6. EFFECT ON ORGAN WEIGHTS of estrogen administration 10 days before x-irradiation, with 500 r. Each point represents the average of values obtained on 5 to 7 CF<sub>1</sub> male mice.

over, lymphoid tissues in the two groups exhibit the same involution after irradiation. Presence of the adrenals is clearly not essential for the estrogen protective action. It is of interest that significantly greater protection is obtained in adrenalectomized animals receiving an adrenal cortical supplement in addition to sodium chloride than in animals receiving only sodium chloride in their drinking water. On the basis of these experiments, it would appear that a certain level of adrenal cortical steroids is necessary, either directly or indirectly, for the maximal estrogen effect. It should be noted that the estrogen per se is more toxic to adrenalectomized animals main-

tained only with sodium chloride than is sesame oil. Twenty-one of 60 adrenalectomized salt-maintained mice succumbed within 10 days of estrogen injection as compared to 8 of 60 similar mice injected with sesame oil. In addition, the operated mice

TABLE 2. INFLUENCE OF ALPHA-ESTRADIOL BENZOATE ON SURVIVAL OF ADRENALECTOMIZED IRRADIATED CF1 MICE<sup>1</sup>

EXPERIMENT NO.	GROUP	TREATMENT	SEX	RADIATION DOSE, r	NO. MICE	PER CENT SURVIVAL—WEEKS AFTER X-IRRADIATION			
						1	2	3	4 <sup>2</sup>
1	Intact	Sesame oil	M	500	66	83	35	24	24
	Intact	Estradiol	M	500	22	86	82	82	82
	Adrenalectomized	1% NaCl, ACE <sup>3</sup>	M		18	95	95	89	82
	Adrenalectomized	1% NaCl, ACE <sup>3</sup> sesame oil	M	500	44	83	27	23	23
	Adrenalectomized	1% NaCl, ACE <sup>3</sup> estradiol	M	500	50	86	78	75	70
2	Intact	Sesame oil	F	550	30	97	23	20	20
	Intact	Estradiol	F	550	27	96	85	81	81
	Adrenalectomized	1% NaCl, sesame oil	F		19	100	95	90	79
	Adrenalectomized	1% NaCl, estradiol	F		16	88	88	75	75
	Adrenalectomized	1% NaCl, sesame oil	F	550	33	51	24	21	18
	Adrenalectomized	1% NaCl, estradiol	F	550	23	70	43	43	39

<sup>1</sup> Estradiol benzoate (0.166 mg. I.M.) injected 10 days before x-irradiation. Controls received equivalent volume of sesame oil. <sup>2</sup> Bold face figures are significantly different ( $P < .05$ ) from the 4-week survival of the appropriate irradiated controls. <sup>3</sup> Adrenal cortical extract (Wilson) 0.2 cc. subcutaneously daily.

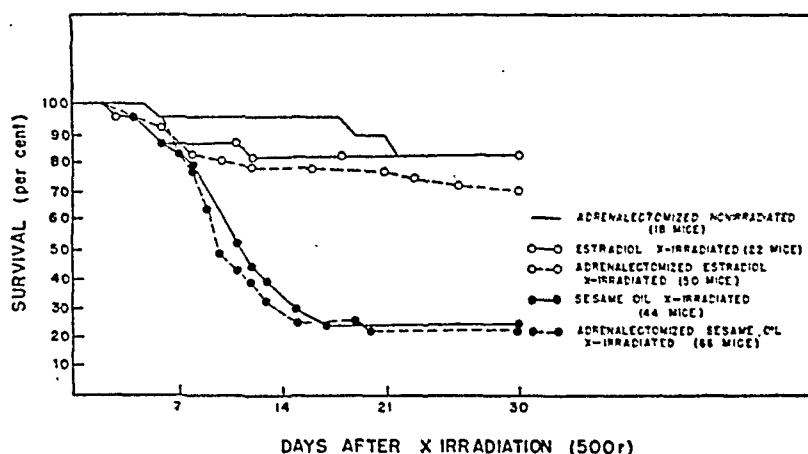


Fig. 7. EFFECT OF ADRENALECTOMY on the protective action of estradiol against x-irradiation of CF1 male mice.

without adrenal cortical supplements appear to be somewhat more sensitive to irradiation than the intact animals.

Significantly, the maximal estrogen protective effect is observed when mice are irradiated during their leukopenic period. This relationship, which is presented graphically in figure 8, along with the observation of a more rapid recovery of the heterophils

in irradiated mice pretreated with estradiol, suggests the possibility that estrogen stimulation renders myelopoietic tissues more resistant to x-rays, perhaps because

TABLE 3. EFFECT OF PRETREATMENT WITH BENZESTROL—2,4-DI (PARAHYDROXYPHENYL) 3-ETHYL HEXANE—ON SURVIVAL OF CFI MICE AFTER X-IRRADIATION

EXPERIMENT NO.	TREATMENT <sup>1</sup>	SEX	RADIATION DOSE, r	NO. MICE	PER CENT SURVIVAL—WEEKS AFTER IRRADIATION			
					1	2	3	4 <sup>2</sup>
1	Controls	F	550	20	90	30	25	25
	Benzestrol, 0.1 mg.	F	550	20	95	80	70	<b>70</b>
	Benzestrol, 0.2 mg.	F	550	20	100	85	85	<b>85</b>
2	Controls	M	550	19	100	5	0	0
	Benzestrol, 0.2 mg.	M	550	15	93	20	13	13
3	Controls	M	500	18	100	44	17	17
	Benzestrol, 0.5 mg.	M	500	20	85	65	55	<b>55</b>
	Benzestrol, 1.0 mg.	M	500	20	95	85	85	<b>85</b>

<sup>1</sup> All injections intramuscularly 10 days before x-irradiation. Controls received an equivalent volume of sesame oil. <sup>2</sup> Bold face figures are significantly different ( $P < .05$ ) from the 4-week survival of the appropriate irradiated controls.

TABLE 4. EFFECT OF PRETREATMENT WITH ALPHA ESTRADIOL BENZOATE, PROGESTERONE AND TESTOSTERONE PROPIONATE ON SURVIVAL OF MALE CFI MICE AFTER X-IRRADIATION (500 r)

EXPERIMENT NO.	TREATMENT <sup>1</sup>	NO. MICE	PER CENT SURVIVAL—WEEKS AFTER IRRADIATION			
			1	2	3	4 <sup>2</sup>
1	Controls, 0.1 cc. sesame oil	41	97	44	32	32
	Estradiol, 0.166 mg.	40	93	85	85	<b>85</b>
2	Controls, 0.1 cc. sesame oil	18	78	33	28	28
	Progesterone, 0.1 mg.	18	83	50	50	50
	Progesterone, 0.2 mg.	18	89	61	55	55
	Progesterone, 0.2 mg. +	18	100	100	100	<b>100</b>
	Estradiol, 0.166 mg.					
	Estradiol, 0.166 mg.	18	89	89	89	<b>89</b>
3	Controls, 0.1 cc. sesame oil	29	87	76	72	72
	Testosterone, 0.1 mg.	30	100	79	66	66
	Testosterone, 1.0 mg.	30	100	59	56	56

<sup>1</sup> All injections I.M. 10 days before x-irradiation. <sup>2</sup> Bold face figures are significantly different ( $P < .05$ ) from the 4-week survival of the appropriate irradiated controls.

these tissues are in a proliferative phase during or shortly after the irradiation. This point is receiving further consideration in current studies in which the histologic appearance of myeloid tissues and the cell population of bone marrow smears are being investigated.

Brues (6) has reported that similar lymphopenias are observed in two species (rabbit and guinea pig) with widely different sensitivities following x-irradiation with 200 r. The heterophils, on the other hand, are decreased markedly only in the guinea pig with this dose ( $LD_{50}$ ). As the radiation dose is increased, a marked depression in heterophils appears in the rabbit as its lethal dose is approached. Brecher *et al.* (7) have observed that lymphocytes and heterophils are decreased rather equally in mice

TABLE 5. EFFECT OF PRETREATMENT WITH ALPHA ESTRADIOL BENZOATE ON SURVIVAL OF MALE CFI MICE AFTER POISONING WITH NITROGEN MUSTARD—METHYL (2,2'-DICHLORO) DIETHYL AMINE HYDROCHLORIDE—3.5  $\mu$ G/GM. SUBCUTANEOUSLY

TREATMENT <sup>1</sup>	NO. MICE	PER CENT SURVIVAL—DAYS AFTER NITROGEN MUSTARD					
		3	4	5	6	8	15
Controls, 0.1 cc sesame oil	31	100	64	29	16	16	16
Estradiol, 0.083 mg.	28	100	57	32	11	11	11
Estradiol, 0.166 mg.	23	91	65	17	9	4	0

<sup>1</sup> All injections intramuscularly 10 days before nitrogen mustard poisoning.

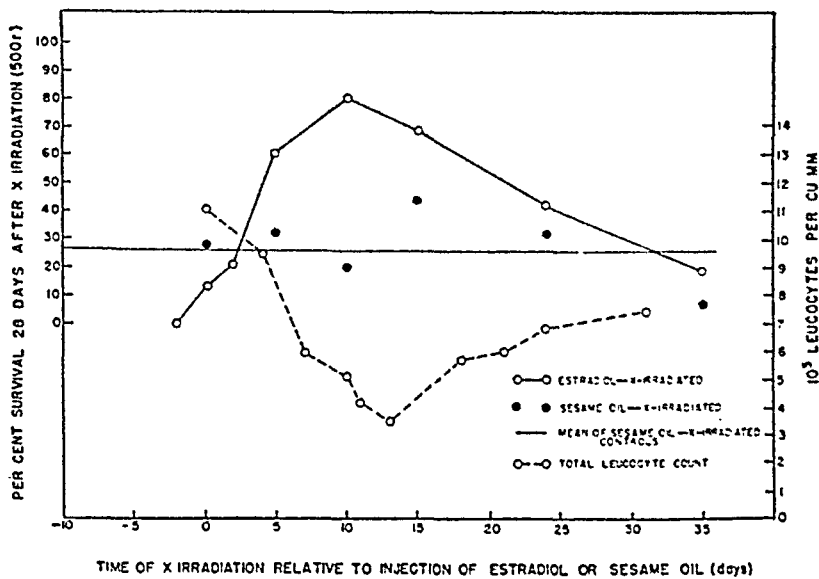


Fig. 8. RELATIONSHIP OF THE ESTROGEN protective effect to the change in total leucocyte count induced by a single injection of estradiol benzoate (EDB).

after an apparently non-lethal exposure to x-rays (400 r). However, their data reveal that there is a fairly rapid recovery of the heterophils at this dose. That there is some correlation between heterophil response and lethal effect is also indicated in our finding that a rapid recovery of heterophils but not of lymphocytes occurs in mice treated with estradiol 10 days before irradiation.

The observation that the anemia of radiation is less severe in the animals conditioned with estrogen may also be significant in explaining the improved survival. Jacobson (8) has reported that male and female mice treated with repeated small doses of estradiol benzoate (16  $\mu$ g. weekly) sufficient to produce a partial replacement of the bone marrow by endosteal new bone formation exhibit an anemia of question-

able significance and no appreciable alteration in the other blood constituents. The absence of frank anemia under these conditions may be due to the fact that some hyperplastic marrow exists between the spicules of invading bone and that, in addition, extramedullary hemopoiesis is more marked than normal in the spleen and liver (8). Although the single large injection of estradiol employed in our experiments did not influence erythrocyte levels in the nonirradiated mice, it is possible that hemopoietic tissue is, nevertheless, rendered more resistant to irradiation as a result of the estrogen or that extramedullary hemopoiesis becomes more evident under these conditions in response to destruction of the marrow by x-rays.

#### SUMMARY

The observation of Treadwell and her co-workers that pretreatment with estradiol benzoate improves the survival of male mice after lethal x-irradiation has been confirmed. It has been demonstrated further that the estrogen protective effect occurs in female mice as well and that Benzestrol, but not progesterone and testosterone, is also effective. The time of injection of estrogen relative to the time of irradiation is critical, for estradiol is most effective when it is given 10 days before the exposure. Estrogen injection 5 or 15 days before irradiation also protects but administration at other intervals fails to increase survival and, indeed, may even potentiate toxicity. Estradiol in dosage sufficient to protect against x-rays does not influence survival after poisoning with a nitrogen mustard.

Estradiol (0.166 mg/mouse I.M.) increases adrenal weight and accelerates thymic involution but has no appreciable effect on weight of the spleen, inguinal nodes or kidneys. Although the increase in adrenal weight after x-irradiation is somewhat greater in mice pretreated with estrogen, the radiation-induced involution of spleen, thymus and inguinal nodes is not altered by the treatment. Experiments are cited which indicate that presence of the adrenals is not essential for the protective action of estrogens.

A leukopenia with maximal depression around 10 to 14 days after estrogen injection has been observed. The reduction in heterophils is more marked and somewhat more consistent than the lymphocyte response. Little change is noted in erythrocyte count following the single injection of estradiol. Leucocytes are decreased to the same levels in both the estrogen-treated and control irradiated animals, although maximal depression and recovery occur earlier in the former. The more rapid recovery of leucocytes in irradiated mice which receive a prior injection of estrogen resides in the heterophil component. The anemia of radiation is also less severe in the estrogen-treated animals.

Significantly, the maximal estrogen protective effect is observed when mice are irradiated during their leukopenic period. That there is some correlation between heterophil response and lethal effect is indicated in our finding that a rapid recovery of heterophils but not of lymphocytes occurs in mice treated with estradiol 10 days before x-irradiation. The possibility is considered that estrogen stimulation renders myelopoietic tissue more resistant to x-rays, perhaps because these tissues are in a proliferative phase during or shortly after the irradiation.

The authors gratefully acknowledge the technical assistance of Miss Eugenia Jackson.

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# RELATION OF FOOD INTAKE TO GROWTH-DEPRESSING ACTION OF NATURAL AND ARTIFICIAL ESTROGENS<sup>1</sup>

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**B**OTH natural and artificial estrogens have been demonstrated to depress growth in rats and mice (1-3). The mechanisms through which this is accomplished have not been adequately clarified. It has been suggested that estrogens may reduce anterior pituitary secretion of growth hormone (2, 3) and thyrotrophic hormone (4), or increase the secretion of adrenocorticotrophic hormone (5), any of which could result in growth depression. Non-pituitary channels may also be involved since it has been shown that estrogens can reduce body weight in hypophysectomized rats (6), inhibit important enzyme systems in the body (7) and possibly exert inhibitory effects on the nervous system (8).

The relation of estrogens to appetite has been largely overlooked, and only a few fragmentary reports have appeared on this subject. Cameron *et al.* (9) noted that rats fed estradiol appeared to eat less food, while Noble (10) observed that estrogens may reduce fluid intake in rats. The artificial estrogens, benzestrol, diethylstilbestrol and hexestrol have been reported to inhibit appetite, respectively, in normal rats (11), alloxan diabetic rats (12) and sheep (13). In goats, Meites and Turner (14) found that a dose of diethylstilbestrol which was just sufficient to induce a definite reduction in daily milk yield also elicited a definite decrease in food and water intake.

The purpose of these experiments was to determine *a*) to what extent estrogen-induced depression of growth rates in rats was due to a voluntary reduction in feed consumption and *b*) whether there were any differences in this respect between an artificial estrogen, diethylstilbestrol, and two natural estrogens, estrone and estradiol.

## METHODS

One hundred female albino rats of the fast-growing Michigan State College strain and 30 female albino rats of the slower-growing Sherman strain were used in these experiments. The three hormones used, diethylstilbestrol, estrone and estradiol,<sup>2</sup> were dissolved in corn oil and injected in volumes of 0.1 ml. daily for periods of either 30 or 75 days. Diethylstilbestrol was given in dosages of 0.001 mg., 0.01 mg. or 0.1 mg. daily, and estrone and estradiol were given only at the rate of 0.1 mg. daily.

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<sup>2</sup> Diethylstilbestrol, estrone and estradiol were kindly supplied, respectively, by Dr. D. F. Green of Merck and Co., Inc.; Dr. D. W. MacCorquodale of the Abbott Laboratories; and Dr. Erwin Schwenk of the Schering Corporation.

Control rats were injected with 0.1 ml. of blank corn oil daily in order to rule out effects which might be attributable to the oil.

The food and water consumption of each group of rats was accurately measured every day, and body weights were recorded every three days. The same quantities of food and water consumed daily by the diethylstilbestrol-treated rats were given to non diethylstilbestrol-treated rats (pair-fed controls) in order to determine the extent of growth inhibition which could be accounted for by anorexia alone. All

TABLE 1. EFFECTS OF NATURAL AND SYNTHETIC ESTROGENS ON FOOD INTAKE AND BODY GROWTH OF RATS

NO. OF RATS	TREATMENT	DAYS	AV. ORIG. BODY WT.	AV. FINAL BODY WT.	AV. INCREASE IN BODY WT.	AV. DAILY FOOD INTAKE	AV. DECR. IN FOOD INTAKE
			gm.	gm.	gm.	gm.	%
Michigan State College Rats—Diethylstilbestrol Series							
			-I-	-I-			
10	Controls—fed <i>ad lib.</i>	30	145.0 ± 5.10	193.0 ± 6.41	48.0	10.9 ± 0.23	
10	0.001 mg. stilb. daily	30	143.5 ± 4.87	174.0 ± 5.93	30.5	9.7 ± 0.32	11.0
10	Pair-fed controls	30	143.0 ± 4.72	175.0 ± 6.30	32.0	9.7	11.0
10	0.01 mg. stilb. daily	30	143.8 ± 5.02	156.5 ± 5.01	13.7	8.8 ± 0.40	19.2
10	Pair-fed controls	30	143.5 ± 3.81	162.5 ± 4.42	19.0	8.8	19.2
10	0.1 mg. stilb. daily	30	154.0 ± 6.44	159.0 ± 6.22	5.0	7.8 ± 0.57	28.4
10	Pair-fed controls	30	149.5 ± 5.65	164.0 ± 4.06	14.5	7.8	28.4
Michigan State College Rats—Natural Estrogen Series							
10	Controls	75	147.0 ± 4.09	186.0 ± 6.01	223.0 ± 7.15	39.0 <sup>2</sup> 76.0	10.4 ± 0.19
10	0.1 mg. estrone daily	75	148.5 ± 3.37	184.0 ± 4.92	201.0 ± 5.97	35.5 52.5	10.6 ± 0.14
10	0.1 mg. estradiol daily	75	146.0 ± 3.15	185.0 ± 3.01	202.0 ± 3.89	39.0 56.0	11.0 ± 0.16
Sherman Rats							
10	Controls	30	137.0 ± 5.32	182.5 ± 4.76	45.5	10.2 ± 0.30	
10	0.1 mg. estrone daily	30	138.5 ± 4.87	167.0 ± 7.03	28.5	9.6 ± 0.29	5.8
10	0.1 mg. stilb. daily	30	135.5 ± 4.53	143.0 ± 6.35	7.5	8.0 ± 0.29	21.5

-I- Standard Error of the Mean =  $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

<sup>1</sup> Average body weight at 30 days.      <sup>2</sup> Average increase in body weight at 30 days.

animals were maintained in an air conditioned room at a constant temperature of 74 degrees Fahrenheit.

RESULTS

The data are summarized in table 1. The average daily water consumption is not included since it closely paralleled food intake in all cases. It can be seen that the largest dosages of diethylstilbestrol used, 0.1 and 0.01 mg. daily, elicited the greatest inhibition of growth and appetite (figs. 1 and 2). The growth and appetite inhibiting

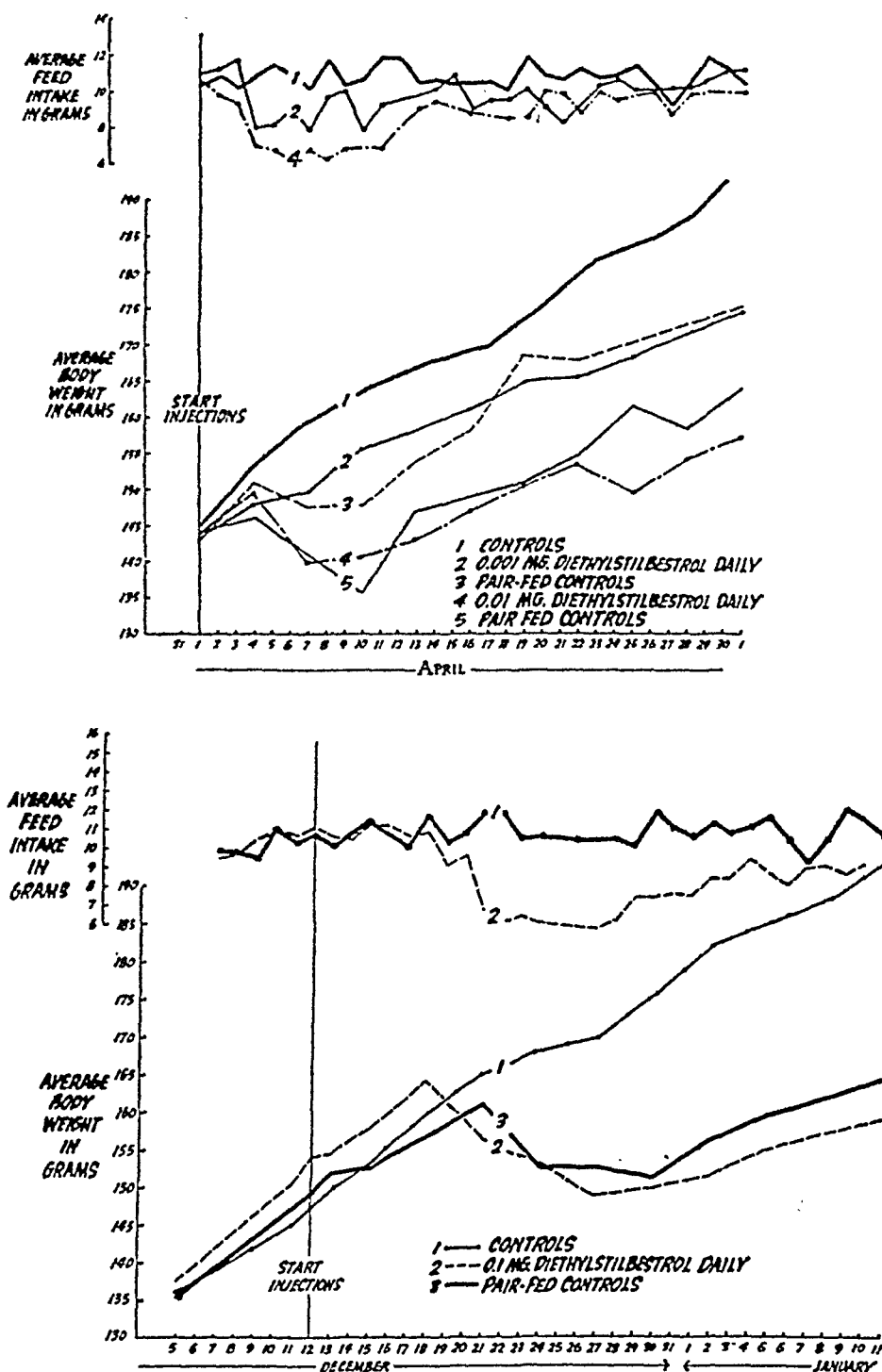


Fig. 1 (upper). EFFECTS OF INJECTIONS of 0.001 mg. and 0.01 mg. of diethylstilbestrol daily on growth rate and food intake of Michigan State College rats. Note the direct relationship between hormone dosage, growth rate and food intake, and the parallel growth rates of the pair-fed controls.

Fig. 2 (lower). EFFECTS OF INJECTIONS of 0.1 mg. of diethylstilbestrol daily on growth rate and food intake of Michigan State College rats. Note the parallel growth rate of the pair-fed controls.

effects of diethylstilbestrol were generally more drastic during the first two weeks than during the last two weeks of the experiment. The growth rate of the pair-fed control groups, limited in each case to the same quantities of food and water con-

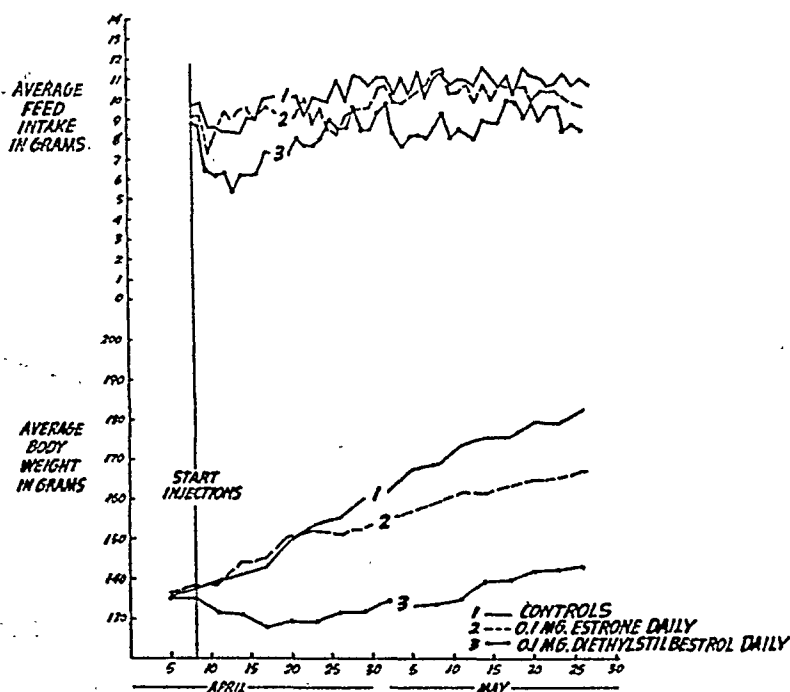
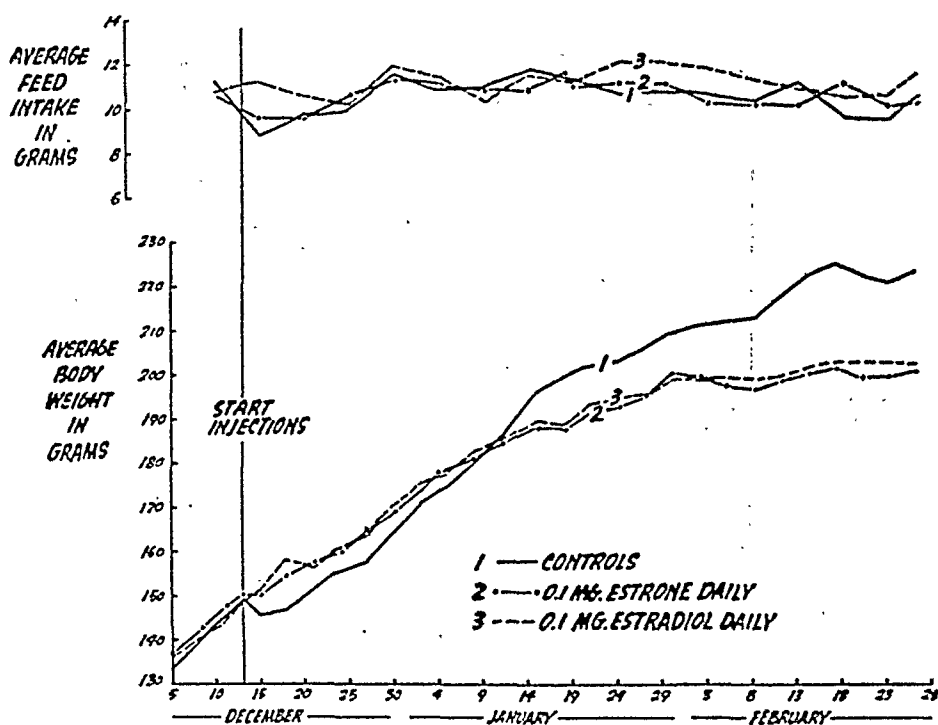


Fig. 3 (upper). EFFECTS OF INJECTIONS of 0.1 mg. of estrone and estradiol daily on growth rates and food intake of Michigan State College rats. Note that the growth rate of these rats was not reduced until after about 30 days of estrogen treatment, and that food consumption was not decreased at any time.

Fig. 4 (lower). EFFECTS OF INJECTIONS of 0.1 mg. of estrone and diethylstilbestrol daily on growth rate and food intake of Sherman rats. Note that diethylstilbestrol induced almost immediate decreases in growth rate and appetite, whereas estrone exerted a delayed and lesser growth-inhibiting effect without significantly altering food intake.

sumed by each of the diethylstilbestrol-treated groups, approximately paralleled the growth rate of the latter. This indicates that most of the growth depression exerted by the three levels of diethylstilbestrol can be accounted for on the basis of reduced food and water intake alone.

In the natural estrogen series (fig. 3) the rats which received 0.1 mg. of estrone or estradiol daily showed no reduction in growth rate until after 30 days, and no decrease in appetite throughout the entire 75 days of the experiment. It is obvious that the growth-inhibiting effects of the least amount of diethylstilbestrol used, 0.001 mg., were more marked than 100 times that quantity of either of the two natural estrogens. These data indicate that growth reduction can be induced with natural estrogens without lowering daily food intake.

The comparative effects of estrone and diethylstilbestrol on the slower growing Sherman rats are illustrated in figure 4. Here, as in the faster growing Michigan State College rats, the growth-inhibiting action of diethylstilbestrol was more drastic and occurred earlier than in the estrone-treated animals. An average daily reduction in food consumption of 21.5 per cent was induced by the diethylstilbestrol, while a small and probably insignificant decrease of 5.8 per cent occurred in the estrone-treated rats.

#### DISCUSSION

The results of these experiments point to the conclusion that diethylstilbestrol can curtail growth (and perhaps gonadal function and lactation) in rats principally by decreasing appetite, while natural estrogens can inhibit growth without any corresponding decrease in appetite. The possibility remains that larger doses of natural estrogens than those used in these experiments could depress food intake, or that rats of different age groups may be more susceptible to the natural estrogens. Some preliminary experiments by the author also indicate that ovariectomized rats are much more responsive than intact rats to the growth-depressing action of estrone, but there is no alteration in daily food intake. It is apparent that factors other than decreased appetite are involved in explaining reduced growth in rats given natural estrogens.

It is interesting to consider how diethylstilbestrol depresses appetite in rats. Both indirect and direct effects seem possible. In goats, Meites and Turner (14) demonstrated that the lactation-inhibiting action of diethylstilbestrol could be completely overridden by the simultaneous administration of thyroxine. This might be considered as indirect evidence that the artificial estrogen reduces thyroid hormone secretion via the pituitary, which in turn results in decreased metabolism and food intake. However, it has also been shown that a restricted food allowance itself is followed by reduced thyroid hormone secretion (15, 16), leaving unanswered the question as to whether appetite or thyroid depression comes first.

Another hypothesis has been suggested by Meites and Turner (14) to explain the reduction in food consumption following administration of artificial estrogens to lactating goats, namely that a vitamin deficiency may be created thereby. These workers noted that the decrease in milk yield following hexestrol administration could be counteracted by feeding extra vitamins in the form of fresh-cut green grass.

Ershoff *et al.* (17, 18) also observed that in immature rats fed massive doses of alpha estradiol, the inhibition of ovarian development could be counteracted by feeding yeast or desiccated whole liver. Preliminary data from our laboratory (19) indicate that the depressing effects of diethylstilbestrol on growth and appetite in rats can be partially overcome by adding vitamin B<sub>12</sub> to the diet, while thiamin and brewer's yeast appear ineffective in this respect.

#### SUMMARY

The relation of appetite to the growth-depressing action of diethylstilbestrol estrone and estradiol was determined in 130 female rats of the Michigan State College and Sherman strains. Diethylstilbestrol was injected in doses of 0.001, 0.01 or 0.1 mg. daily into three groups of rats, and food and water consumption were recorded daily. Three groups of control rats were fed the same amounts of feed and water consumed daily by the diethylstilbestrol-treated animals. The two natural estrogens were injected into several groups of rats in doses of 0.1 mg. daily, and food and water intake were similarly recorded.

All levels of diethylstilbestrol decreased growth and food and water intake, with the larger doses inducing the greater inhibitory effects. The growth rates of the paired control groups paralleled those of the diethylstilbestrol-treated groups, showing that the growth-inhibiting effect of this hormone was due mainly to its ability to depress appetite. The natural estrogens were less effective in reducing growth and did not decrease food consumption. The possible mechanisms through which the natural and artificial estrogens exert their different effects on growth or appetite are discussed.

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# INFLUENCE OF INDUCED HYPO- AND HYPERTHYROIDISM ON VITAMIN E REQUIREMENT OF CHICKS<sup>1</sup>

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THE effects of feeding small amounts of synthetic thyroprotein to chickens are currently being investigated in this laboratory. In one particular trial, it was observed that young cockerels fed a conventional diet supplemented with synthetic thyroprotein spontaneously developed E-deficiency symptoms while birds used as controls remained normal (1). Vitamin E deficiency symptoms are not unusual in growing chickens fed a conventional diet, especially if the fats in the diet become rancid. But in the present case this suggests that the diet supplied marginal amounts of vitamin E and that the mild hyperthyroidism induced by the feeding of synthetic thyroprotein may have increased the birds' vitamin E requirement.

A review of the literature did not yield information on the relationship of thyroidal activity to vitamin E requirement. This was not unexpected because it is rather difficult to obtain definitive vitamin E deficiency symptoms in mammals. The growing chick, however, is an excellent subject for E-deficiency studies because it is relatively simple to produce the characteristic symptoms—ataxia and cerebellar hemorrhage (2, 3). Therefore, any effect of hyperthyroidism on vitamin E requirement would be more readily observed in chicks than in other experimental animals.

The present communication describes the effects of experimentally induced hyper- and hypothyroidism in chicks fed purified diets containing known amounts of vitamin E.

## EXPERIMENTAL

Two hundred four-day-old White Leghorn males were divided at random into 12 groups of 17 chicks, and each group was confined in a separate compartment of an electric battery brooder provided with wire mesh floors. Two groups served as replicates for each of the six diets fed.

Dam's (3) basal diet no. 190<sup>3</sup> was modified to provide the following diets:

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<sup>3</sup> U.S.P. XII Salt Mixture plus 0.0012 gm.  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per 100 gm. of basal diet was used instead of the mixture employed by Dam.

<i>E-deficient</i> <sup>4</sup>	<i>E-sufficient</i>
1. No supplement	4. No supplement
2. 0.04% thyroprotein <sup>5</sup>	5. 0.04% thyroprotein <sup>5</sup>
3. 0.1% thiouracil	6. 0.1% thiouracil

The dry material was mixed daily with lard so as to provide a 30 per cent lard mixture and all unconsumed food was discarded before the fresh supply was put into the feeding trough each day. Vitamins A and D were administered twice weekly by dropper at the rate of 1200 IU of A, and 150 IU of D<sub>3</sub> per chick per week.

The experimental period was 28 days. Observations were made on time of onset of ataxia, time of death and at autopsy, the presence of cerebellar hemorrhage was determined.

### RESULTS

It may be seen in table 1 and figure 1 that there was a definite relationship between thyroid state and the time and incidence of death of the E-deficient chicks.

TABLE 1. EFFECT OF HYPER- AND HYPOTHYROIDISM ON VITAMIN E REQUIREMENT OF CHICKS

DIET	SUPPLEMENT	CUMULATIVE NO. DEAD ON DAY:				TOTAL MORTAL- ITY	MEAN AGE AT DEATH	AVERAGE BODY WEIGHT	
		7	14	21	31			Day 1	Day 28
						%	days	gm.	
Basal E-deficient	Thyroprotein (0.04%)	0	15	22	24	71	15	41	143
	None	0	4	13	18	53	20	42	170
	Thiouracil (0.1%)	0	0	3	14	41	28	42	145
Basal, plus 10 mg. vit. E/100 gm. diet	Thyroprotein (0.04%)	0	0	0	5 <sup>1</sup>	15 <sup>1</sup>	28 <sup>1</sup>	41	183
	None	0	0	0	0	0		41	194
	Thiouracil	0	0	0	0	0		42	193

<sup>1</sup> Severe perosis but no symptoms of E-deficiency; see text.

The total mortality from E-deficiency was highest in the hyperthyroid group, intermediate in the euthyroid group and lowest in the hypothyroid group. The mean age at death was 15, 20, and 28 days, respectively, for each of these groups, indicating the relative rate of depletion of the vitamin E stores. In fact, 48 per cent of the hyperthyroid chicks were dead before any of the animals in the other groups had started to show deficiency symptoms, and 35 per cent of the euthyroid chicks were dead by the time any of the hypothyroid chicks died. All of these values are statistically significant at the 1.0 per cent level.

All of the E-deficient chicks that died became ataxic, and at autopsy exhibited cerebellar hemorrhage. On the other hand, none of the E-sufficient chicks showed any symptoms of encephalomalacia, either when alive or at autopsy. The only deaths in these groups occurred in the hyperthyroid chicks, and, since those that died exhibited such severe perosis in both legs that they did not have ready access to food

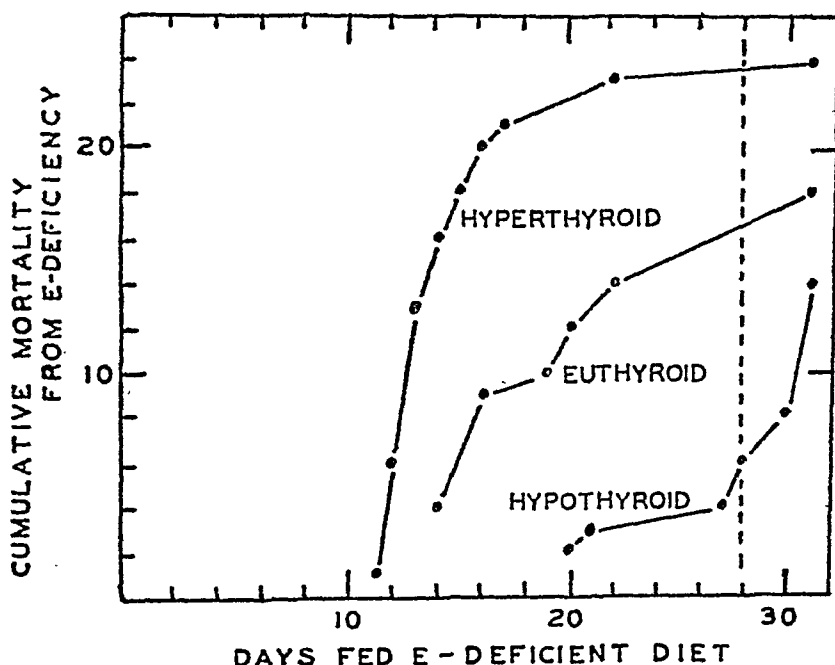
<sup>4</sup> Ten mg. alpha tocopherol acetate (Merck) was added for each 100 gm. of basal diet.

<sup>5</sup> Thyroprotein contains 3.0 per cent thyroxine according to the manufacturer's chemical assay.

and water, it is believed they died of starvation. In all, 29 per cent of the E-sufficient, hyperthyroid chicks developed perosis (a condition in which the Achilles tendon slips from the condyles of the tibiotarsal-tarsometatarsal joint). Perosis also occurred in the hyperthyroid, E-deficient chicks but the incidence was only 3.0 per cent. Perhaps the reason for such a low incidence in this group is that most of the chicks died too early in the course of the experiment to develop the disorder.

#### DISCUSSION

Precedence for the idea that thyroid state affects the rate of depletion of vitamin stores and thus vitamin requirement is established in the review of Drill (4). It has



vitamin E: soybean (or corn) oil and 'crude' casein. Furthermore, the time interval (one week) was of too short duration to sufficiently deplete the endogenous vitamin. Dam (3) was unable to produce deficiency symptoms in less than 12 days, and in the present study the first chicks did not show symptoms until the end of the second week on a deficient diet.

The excessive incidence of perosis which occurred in hyperthyroid chicks fed the E-sufficient diet suggests that elevated metabolism may also increase the requirement for other substances. Manganese (6), choline (7) or biotin (8, 9) deficiency is known to cause perosis.

One of the most interesting implications of this study lies in its possible use in the biological assay of vitamin E. The use of hyperthyroidism to accelerate depletion of endogenous vitamin E would make possible the employment of 'basal' animals for assay purposes in the manner used by Robblee *et al.* (10) in studying an unidentified chick growth factor. These workers found that synthetic thyroprotein increased the requirement for the growth factor and hence could be used to produce depleted animals for test purposes.

#### SUMMARY

Experimental hyperthyroidism increases, whereas induced hypothyroidism decreases the rate of utilization (depletion) of endogenous vitamin E in chicks fed an E-deficient diet. There is some evidence that hyperthyroidism may also increase the requirement for choline, biotin or manganese. The findings of the present work imply that supplementary vitamin E may be indicated in natural or induced hyperthyroidism, or in cases of thyroid therapy. The technique used in this study would appear to be valuable in the biological assay of vitamin E. Hyperthyroidism, used to accelerate depletion of endogenous vitamin E, would make possible the employment of 'basal' animals for assay purposes.

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# EFFECT OF SEVERE STRESS UPON THYROID FUNCTION

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MANY factors are known to increase the metabolic rate; among these are exercise, extremes of temperature, insomnia, food, fever and a great variety of emotional disturbances. The mechanisms by which the increases are effected have been incompletely elucidated. One of the probable contributory factors which was investigated early was the rôle of adrenalin. Krantz and Means (1) found that a single subcutaneous injection of 0.625 mg. of adrenalin raised the metabolism of normal people to a level of about plus 25 per cent. Marine and Baumann (2) observed in the rabbit and cat with an intact thyroid that a transient symptom-complex was produced by injury to the adrenals, which closely resembled Graves' disease. More recently, Soffer and associates (3) found that prolonged administration of adrenalin to dogs was followed by hyperplastic changes in the thyroid. Bioassay of the serum of thyroidectomized dogs after adrenalin administration showed an increase in circulating thyrotropin. Although adrenalin probably exerts a calorogenic effect, independent of changes induced in the thyroid, the latter are doubtless of significance under certain circumstances.

The influence of the cervical sympathetic nervous trunk upon the thyroid was investigated by Cannon, Binger and Fitz (4) by anastomosing the distal end of the trunk with the phrenic nerve. There resulted an increased oxygen consumption, nervousness, weight loss and cytological changes of hyperthyroidism. Friedgood and Cannon (5) upon investigating the phenomenon concluded that the thyroid stimulation was mediated through the pituitary. Uotila (6) found that extirpation of various parts of the cervical sympathetic system failed to produce much direct effect on the cytology or function of the thyroid.

Uotila (7) as well as Wolf and Greep (8) reported that exposure of rats to cold increased the activity of the thyroid, provided that the pituitary was present. Uotila showed that it was necessary also to have the hypophyseal stalk intact.

Starr and Roskelly (9) observed that in rats exposed to temperatures of 12° to 17° C., the thyroid cells initially became hypertrophied but later decreased markedly in size when the exposure was extended to 56 days. Dempsey and Astwood (10) concluded on the basis of indirect evidence that the release of thyroxin from the thyroid increased when the animal was exposed to cold and decreased when he was in a heated environment.

Leblond and collaborators (11), using radioiodine to study thyroid function, concluded that in rats exposed to cold (0° to 2° C.) for various intervals there was questionable stimulation of the thyroid within the first 3 days, definite after 7 days, maximal at 26 days, and none at 40 days. At the time of maximal stimulation by the cold the fixation of radioiodine was 2.7 times that of the control.

Exposure to heat was found to lessen thyroid activity. The decrease was observed as early as after one day, and it persisted for at least 26 days.

We have investigated in rats, the effect of starvation, heat, cold, adrenalin, adrenal cortical extract, typhoid vaccine, and trauma upon the distribution of radioiodine in the thyroid gland and in the serum. The content of radioiodine in urine and the total and protein-bound radioiodine of serum were determined in a series of patients while they were undergoing pronounced stress, and constitute a separate report.

#### METHODS

The rats were of the Sprague-Dawley strain, and almost all of them were male. They were raised in the Thorndike Memorial Laboratory and were maintained on a diet of Purina Laboratory Chow, supplemented weekly with carrots and lettuce. At the time of the various experiments the rats were from 60 to 70 days of age. From 4 to 6 animals were used to test any one phase of each experiment.

The tracer dose of radioiodine ( $I_{131}$ ) consisted of 100 microcuries, except where otherwise stated. In all instances it was given subcutaneously, usually in 1 cc. of saline. Caution was taken to pinch the skin at the site of injection very firmly in order to prevent leakage. The rats were killed by a blow on the head, except in the ones from which blood was taken; these were killed by a guillotine method. In one experiment sodium pentothal anesthesia was used but varying degrees of shock caused such marked discrepancy in the results that they were unreliable. The thyroid glands were removed quickly, weighed upon a torsion spring balance, digested in 20 per cent potassium hydroxide and analyzed for the radioiodine content. The determination of radioiodine in serum consisted of placing 0.5 or 1 cc. of serum in a bottle cap with one drop of dupanol. After drying in an oven the cap was placed under a Geiger-Müller tube and counts were made. In determining the PBI<sup>1</sup> 1 cc. of serum was pipetted into a 15 cc. centrifuge tube. Then 9 cc. of 10 per cent trichloroacetic acid was added while the solution was stirred vigorously. After centrifugation the supernatant fluid was decanted. The precipitate was washed three times with 5 cc. portions of 10 per cent trichloroacetic acid, transferred quantitatively to bottle caps, dried at 100°C. and then isotope counts were made.

Animals exposed to cold were kept at 5° C., while those exposed to heat were kept at 38° C.; the other rats were kept at 26° C. Other conditions of the experiments are described as each is presented.

*Experiments I and II.* Each animal was given a tracer dose of radioiodine one hour before it was killed. It can be observed in figure 1 that in animals that had been fasted, exposed to cold or heat, or given adrenalin<sup>2</sup>, the concentration of radioiodine in the thyroid gland was less than normal. The changes were greater in animals subjected to heat or starvation for two days than in those so treated for three days. The rats given adrenalin one hour before radioiodine was injected, and again at the time the radioiodine was given, had a lower concentration of isotope in the thyroid than did the ones given adrenalin only with the radioiodine.

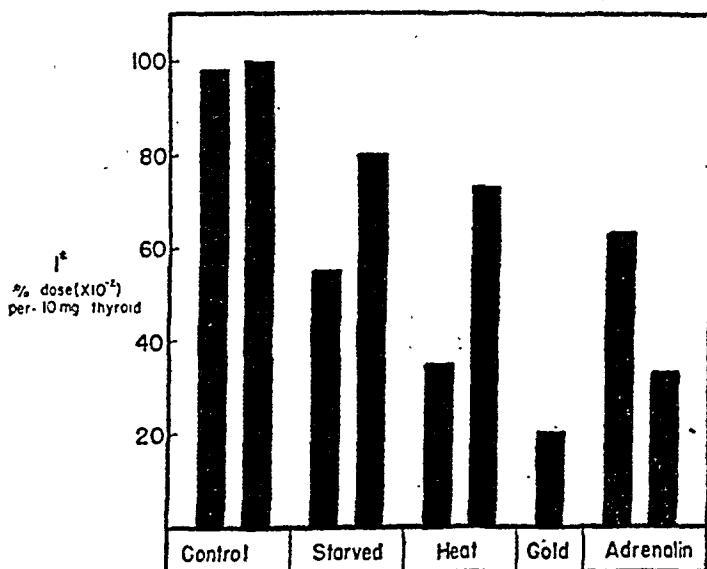
<sup>1</sup> Protein bound iodine.

<sup>2</sup> The solution of aqueous adrenalin contained 1 mg/cc. The oily preparation contained 2 mg/cc.

*Experiment III.* In this experiment each animal was killed 2 hours after radioiodine was injected. The rats were fasted for 3 days, or exposed to heat or cold for 2 days. One group of normal rats was given radioiodine followed one hour later by 0.2 cc. of adrenalin in oil, and 0.1 cc. of aqueous adrenalin. As shown in figure 2, there was a subnormal concentration of radioiodine in the thyroid following adrenalin therapy, starvation and exposure to heat or cold.

*Experiment IV.* In this experiment, the radioiodine was given 24 hours preceding the death of the rat. No food was given during the last 4 hours of the experiment. One group was given 0.2 cc. of adrenalin 2 hours before death. Another group received the same dosage 48, 24 and 2 hours preceding death. Five rats were subjected to trauma by applying a rubber band on the left hind leg for 15 hours 2 days before death, on the right hind leg for 2 hours on the next day, and on the right front leg for one hour beginning 2 hours before death. At least one leg of each animal became

Fig. 1. RESULTS OF 2 EXPERIMENTS are presented. The 2nd, 4th and 6th columns are results of the 2nd experiment. The only difference in the characteristics of the 2 experiments is that the 2nd one was conducted for 3 days while the first one was for 2 days. One group of rats, shown in the last column, was given 0.2 cc. of adrenalin one hour before radioiodine and again with it; the other group was given 0.2 cc. of adrenalin concomitantly with the isotope. Each column represents average result obtained with from 3 to 6 rats.



gangrenous. Four rats were each given 0.5 million killed typhoid bacilli intraperitoneally 2 days before decapitation; the same sized dose was given the following day, and one million organisms were administered 2 hours before the rats were killed.

Bilateral adrenalectomy was performed on 15 rats 2 days before death; they were given saline as drinking water. Five received no other treatment; 5 were given 0.5 cc. of lipoadrenal extract (Upjohn) at 48, 24, and 3 hours, respectively, before death; 5 were given 0.2 cc. of adrenalin in oil at 48, 24, and 2 hours, respectively, before death.

As seen in figure 3, the normal animals which received adrenalin, trauma or typhoid vaccine were found to have distinctly less protein-bound radioiodine in the serum than did the untreated ones. On the other hand, the concentration of isotope in the thyroid was hypernormal. The studies with the adrenalectomized animals demonstrated less protein-bound radioiodine in the ones treated with adrenal cortical extract or adrenalin than in the controls, but there were no statistically significant differences in the quantity of isotope in the thyroid. All of the adrenalectomized rats had more radioiodine in the thyroid than did normal animals.

*Comments.* The results that have been presented thus far show that following the various types of stress the concentration of radioiodine in the thyroid of normal rats became subnormal when the isotope was given one or two hours before thyroidectomy, but hypernormal when it was administered 24 hours before. The serum protein-bound radioiodine was found to be subnormal in the animals subjected to severe stress and given radioiodine 24 hours preceding death. Since there is cytological evidence (7) of increased thyroid activity within 24 hours of exposure to stress, an increased rate of uptake of radioiodine by the thyroid would be expected.

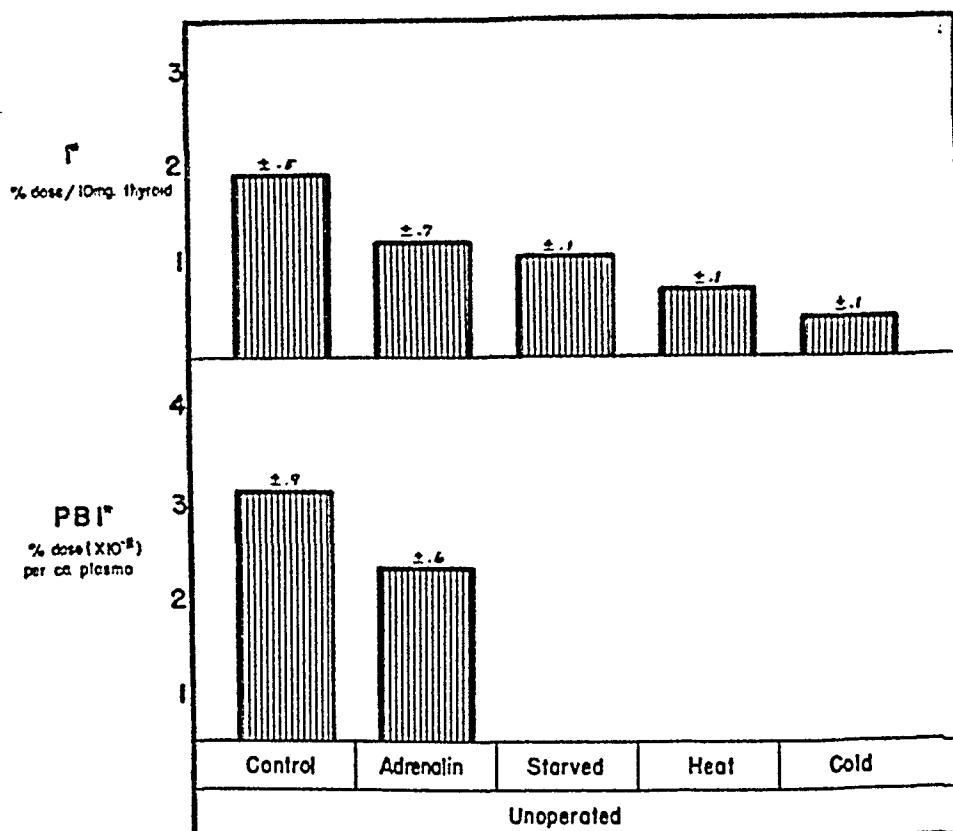


Fig. 2. EACH COLUMN presents the mean value obtained with from 3 to 6 rats; standard deviation from the mean is indicated.

However, with the increased production of thyrotropin there would occur an increased rate of manufacture of thyroid hormone and of its transfer into the blood stream. The increased metabolic rate associated with the severe stress could be expected to increase the rate of breakdown of thyroid hormone. Since the quantity of radioiodine in the thyroid and in protein-bound form in the serum is a balance of the factors discussed above as well as the quantity of radioiodine excreted or existing in sites other than the thyroid or serum, with the information available, it is difficult to evaluate adequately the individual factors involved. In order to help elucidate the problem the following experiment was conducted.

*Experiment V.* Each of 39 rats was injected with 8 microcuries of radioiodine every day for 3 days. They were given 16 microcuries upon the fourth day and killed

on the fifth. All of these animals were fasted for from 15 to 18 hours before death. The thyroid gland of 8 animals was removed, using ether anesthesia; each of these rats was killed 3 hours later. Four of them were given 0.3 cc. of adrenalin in oil immediately after thyroidectomy and 0.2 cc. of aqueous solution of adrenalin 20 minutes before death. The purpose of this phase of the experiment was to determine the effect of adrenalin, and the hypermetabolism which it produces, upon the utilization of the protein-bound radioiodine of the serum. The effect of the operative

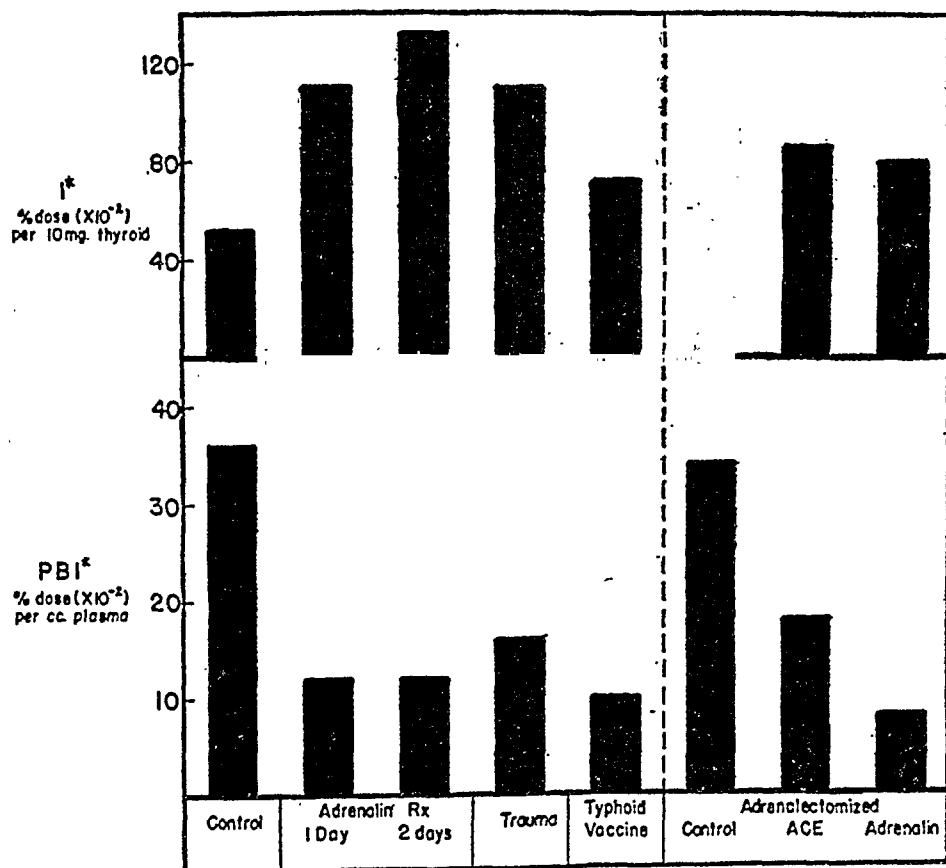


Fig. 3. EACH COLUMN presents mean value obtained with from 4 to 6 rats; standard deviation from the mean is indicated.

procedure upon the thyroid was evaluated by a sham operation. The muscles anterior to the thyroid were cut and the gland was completely exposed.

In order to permit the thyroid to evacuate its hormone during stress but to antagonize additional formation of hormone, 100 mg. of sodium thiouracil was given in 2 cc. of saline, intragastrically, to 8 rats, 5 hours before they were killed. Three hours before death, each of 4 of these animals was given 0.3 cc. of adrenalin in oil; 0.2 cc. of an aqueous solution of adrenalin was given 20 minutes before decapitation. The remainder of the 39 rats served as controls. Determinations were made in each of the 39 rats of the total radioiodine concentration in the thyroid and in the serum, as well as in the protein-bound fraction of the latter.

None of the treatments caused an unequivocal change in the radioiodine content of the thyroid. The total and the protein-bound iodine of the plasma was less in the

two groups of rats given adrenalin than in any of the others. In the group treated with thiouracil it is presumable that synthesis of the hormone had ceased, but it was still possible for adrenalin, by means of increasing thyrotropin production (3), to increase the rate of transfer of thyroid hormone into the blood and to increase the rate of metabolism of protein-bound radioiodine. In the rats that were thyroidectomized, only the latter effect would be anticipated. However, it is quite possible that, with the manipulation of the thyroid gland, more protein-bound radioiodine was liberated from the thyroid than was induced by the increase in thyrotropin. The thyroidectomized control group was not found to have an increase in thyrotropin nor

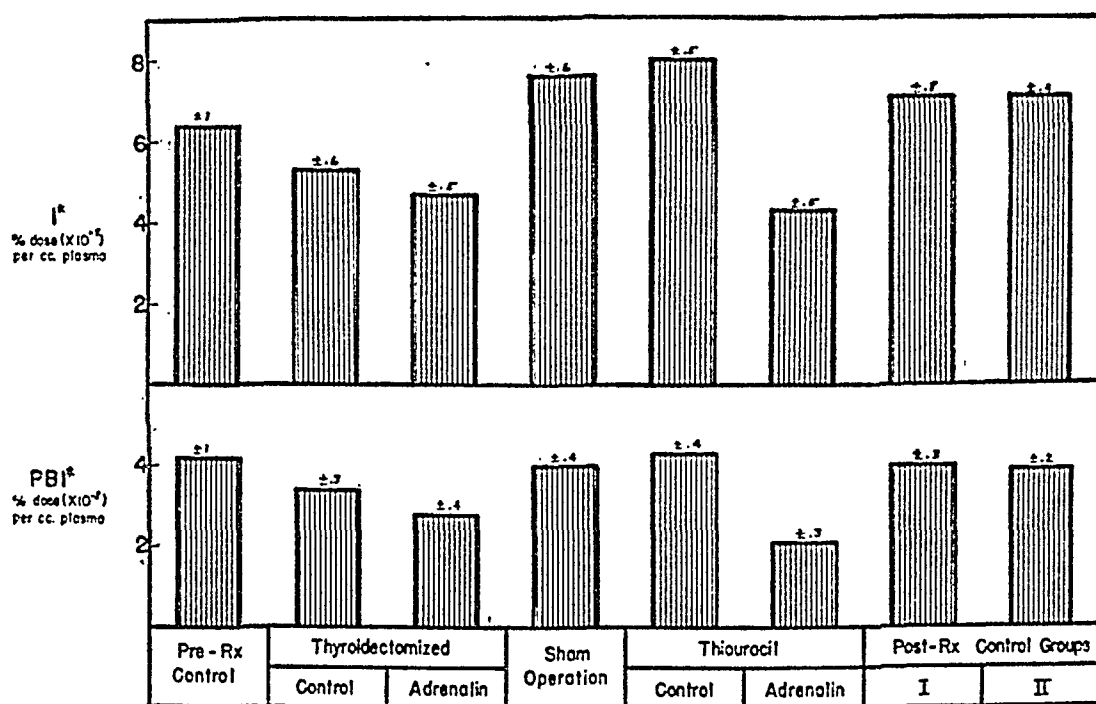


Fig. 4. THE GROUP designated as 'pre-treatment control' were killed just before thiouracil or adrenalin was administered, or thyroidectomy was performed. The rats in the post-treatment control groups were killed simultaneously with the ones that had been treated. Each column presents the mean values obtained with 4 or more rats; standard deviation from the mean is indicated.

in the protein-bound radioiodine. Although it is probable that these animals were given extra adrenalin endogenously, the increased rate of utilization of the thyroid hormone may have helped to balance the increased transfer of 'thyroxin' from the thyroid to the plasma. Just what effect the different forms of treatment had on the excretion of radioiodine or its distribution in other parts of the body was not determined.

#### DISCUSSION

It has been shown that various types of stress affect the quantity of radioiodine in the thyroid and in the serum, particularly the protein-bound fraction. However, it is difficult to determine the extent to which various phenomena are involved in producing the alterations in iodine metabolism. The concentration of isotope in the thyroid is, of course, a balance between the quantity of it taken from the blood by the gland and the amount released.

The rate of these changes depends upon the gradient of iodine between the blood and thyroid, thyrotropic hormone activity, rate of utilization of the thyroid hormone and other factors. Adrenalin increases the rate of release of thyrotropin from the pituitary, as apparently does the exposure to cold. Whether other alarming stimuli increase thyrotropin release has not been proved, although it seems logical that many of them would increase it. Apparently various stresses lead to an increased production of thyroid hormone, but there was also an increased rate of utilization of it. Neither the presence of the adrenals nor of the thyroid was necessary for adrenalin to decrease the protein-bound radioiodine of the plasma.

The results of Leblond and colleagues (11) show that the duration of exposure to cold and the dosage of iodine are important in determining the concentration of isotope in the thyroid. When in their experiments 0.2 microgram of iodine was used, a decrease in the radioiodine in the thyroid was found during the first few days of exposure. When 5 micrograms were used, there was an apparent increase after a seven-day exposure and maximal increase after a 26-day exposure, but no increase after a 40-day exposure.

#### SUMMARY

In rats, the concentration of radioiodine by the thyroid and its distribution in the serum were found to be influenced by adrenalin, trauma and typhoid vaccine. Adrenalin or adrenal cortical extract decreased the quantity of protein-bound iodine in the serum of adrenalectomized animals. Adrenalin decreased it in normal and thyroidectomized rats. Fasting, or exposure to cold (5° C.) or to heat (38° C.) for intervals of three days was associated with a subnormal concentration of radioiodine in the thyroid.

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# METABOLISM OF DOGS DURING INTOXICATION FROM AGENIZED WHITE WHEAT FLOUR<sup>1</sup>

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IT HAS been repeatedly demonstrated that various protein materials which have been treated with nitrogen trichloride ('Agene') when fed to animals will cause signs of intoxication, principally epileptiform convulsions (1, 2). These signs have been adequately described in previous communications (3, 4). The present investigation sought to determine some of the metabolic changes accompanying such intoxication. Feeding experiments upon a variety of animals have demonstrated a marked species specificity for this type of poisoning, and of all animals tested, the dog is outstanding in its susceptibility to the toxic agent. For this reason this animal was employed throughout the present study.

## METHODS

Six healthy, adult, mongrel dogs of either sex, and with body weights from 5 to 9 kg. each, were studied. They were housed in individual metabolic cages kept in an air-conditioned animal house at 73°F. Use of these cages permitted separate quantitative measurement of dietary and fluid intake and of the excretion of urine and feces. The animals were allowed freedom of activity within the cages but not outside.

Diets previously shown to be nutritionally adequate for dogs (3) were fed *ad libitum*. The animals consumed a daily average of 35 grams of food per kilogram of body weight and maintained their body weights well throughout the course of the study. Seventy-five per cent of the diet consisted of white wheat flour, which was untreated during the preliminary control period. During the period of intoxication, the untreated flour was replaced by flour treated with nitrogen trichloride ('Agene') to the extent of 10 grams of  $\text{NCl}_3$  per 100 pounds of flour. The diet was otherwise unchanged.

Metabolic observations were made on two separate days of a preliminary control period which lasted 10 days. During the phase of intoxication, observations were made from day to day after clinical evidence of poisoning became unequivocal. Death usually ensued within 15 days after the introduction of feeding with agenized flour, but no animal was studied while moribund.

Daily records were kept of food and water intake, urine volume and specific gravity, and body weight. On the appropriate days, blood samples were taken from the external jugular vein, with the dog under light intravenous pentobarbital anesthesia. When the animals were judged to be approaching a terminal state, electroencephalographic records were made in a manner previously described (3). This procedure served to confirm the clinical diagnosis of intoxication.

The following qualitative tests were made on the urine of all animals on alternate days throughout the study: reducing substances,  $\text{pH}$ , protein, bile pigments, ketone bodies, blood (benzidine) and microscopic formed elements. The urine was analyzed quantitatively for the following substances: total nitrogen, urea nitrogen, creatinine, calcium and phosphorus. Formed elements of the blood

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<sup>1</sup> The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

were counted and hemoglobin and hematocrit were measured. Samples of whole blood or serum were analyzed quantitatively for glucose, non-protein nitrogen, albumin, globulin, urea nitrogen, phosphorus, calcium, acetylcholine and cholinesterase. Circulating serum acetylcholine was determined by the method of Wait (5), a procedure which uses the isolated heart of the mollusc *Venus mercenaria*. The serum cholinesterase was determined by the method of Michel (6) in aliquots of the same samples of sera as used for estimating acetylcholine. Cholinesterase activity is reported in units of decrease of  $pH$  per hour in a veronal buffer containing acetylcholine bromide as the substrate.

Liver function was evaluated in both groups of animals by three different tests that are widely used clinically; viz., cephalin flocculation, thymol turbidity and bromsulphalein retention.

### RESULTS

Although there was some variation, the average animal commenced to show signs of intoxication which became pronounced by the 7th day. A terminal condition was reached in about 15 days.

TABLE 1. CLINICAL COURSE OF 6 DOGS BEFORE AND DURING INTOXICATION FROM AGENIZED WHITE WHEAT FLOUR COMPARED WITH AVERAGE SERUM ACETYLCHOLINE AND SERUM CHOLINESTERASE

PERIOD	TYPICAL CLINICAL COURSE	VALUES FOR SERUM		
		Acetylcholine $\mu g/100\text{ ml.}$	Cholinesterase units $\Delta pH/hr$	Ratio: acetylcholine/ cholinesterase
Control <sup>1</sup>	Normal behavior	0.87	0.47	2
Intoxication <sup>2</sup>				
Day 4	Intermittent periods reminiscent of 'petit mal.' Few if any convulsions	0.54	0.60	1
Day 7	Typical epileptiform, convulsions about every 4-8 hrs.	0.48	0.59	1
Day 11	Typical epileptiform convulsions about 1/hr.	1.86	0.34	5
Day 15	'Status epilepticus,' convulsions very frequent (no animal studied when moribund)	2.76	0.06	46

<sup>1</sup> Mean of two observations on different days.

<sup>2</sup> Observations on single days during progressive intoxication.

Alterations in the serum cholinesterase activity and in the concentration of acetylcholine in the serum were the only significant metabolic changes found in this study (table 1). The average value for acetylcholine in the control period was 0.87  $\mu g/100\text{ ml.}$  of serum. The concentration of this substance increased progressively, following the appearance of the signs of intoxication, to attain an average final value of 3.60  $\mu g/100\text{ ml.}$  of serum. At the same time serum cholinesterase activity decreased from an average control value of 0.35 units to a final value of 0.06 units. This decrease did not become notable until the clinical signs were well advanced.

The remaining metabolic measurements showed no consistent or biologically significant changes during the period of intoxication. Studies of the formed elements of the blood, as well as of the hemoglobin and the hematocrit, revealed no notable

variations in value between the two periods (table 2A). These values were within the normal limits for healthy dogs. Similarly, there were no striking variations in blood glucose, serum non-protein nitrogen, serum total protein, serum albumin, serum globulin, serum A/G ratio, serum urea nitrogen, serum inorganic phosphorus or serum calcium (table 2B). All of the values for these components of the blood and

TABLE 2. AVERAGE VALUES FOR HEMATOLOGY, BLOOD CHEMISTRY, AND URINARY EXCRETION PRODUCTS OF 6 DOGS BEFORE AND DURING INTOXICATION FROM AGENIZED WHITE WHEAT FLOUR

COMPONENT <sup>1</sup>	CONTROL PERIOD <sup>2</sup>	PERIOD OF INTOXICATION <sup>3</sup>
<i>A. Blood—Hematology</i>		
Erythrocytes, millions/mm. <sup>3</sup>	4.3	4.3
Leukocytes, thousands/mm. <sup>3</sup>	7.4	8.1
Hemoglobin, gm/100 ml.	13.2	13.5
Hematocrit, %	40.3	39.9
<i>B. Serum and whole blood chemistry</i>		
Whole blood glucose, mg/100 ml.	100	81
Serum non-protein nitrogen, mg/100 ml.	35	34
Serum total protein, gm/100 ml.	6.0	6.2
Serum albumin, gm/100 ml.	3.3	3.5
Serum globulin, gm/100 ml.	2.8	2.7
Serum A/G ratio	1.2	1.3
Serum urea nitrogen, mg/100 ml.	11.9	11.7
Serum inorganic phosphorus, mg/100 ml.	5.2	4.7
Serum calcium, mg/100 ml.	13.3	12.9
<i>C. Urine</i>		
Daily volume, ml/day	128	135
Specific gravity, water = 1.000	1.037	1.037
Total nitrogen, gm/day	3.69	3.27
Urea nitrogen, gm/day	1.70	1.65
Creatinine, mg/day	215	187
Calcium, mg/day	14.4	11.9
Phosphorus, mg/day	329	314

<sup>1</sup> None of the measurements listed in table 2 showed consistent or biologically significant differences between the control and intoxication periods.

<sup>2</sup> Average of 2 measurements on separate days of the control period.

<sup>3</sup> Average of 2-4 measurements toward the end of the period of intoxication.

serum were well within the normal limits for healthy dogs. Between the control period and the test period there were no significant changes in the average daily urinary excretion of total nitrogen, urea nitrogen, calcium, phosphorus or creatinine (table 2C).

Kidney function remained normal throughout the period of intoxication. The daily volume of urine and the average specific gravity of the urine did not vary significantly between control and test periods (table 2C), and a well concentrated, slightly alkaline urine was produced at all times. No variations from the normal were

seen at any time in urinary reducing substances, acidity, protein, bile pigments, ketone bodies, free hemoglobin or microscopic formed elements.

Liver function was not disturbed at any time, as judged by normal retention of bromsulphalein in all animals, and by normal results for thymol turbidity. The cephalin flocculation was positive in all specimens of serum during both the control and experimental periods. This 'false positive' result was presumably caused by a serum protein peculiar to the dog, and not found in the serum of healthy human beings.

#### DISCUSSION

Previous reports from this laboratory by Silver *et al.* (3), claiming failure of dogs to concentrate urine normally when suffering from the toxic effects of agenezed protein, seem to be refuted by this study. It is believed by the present authors that this misconception arose from the fact that in the former studies the animals splashed water from their drinking pans into the urine-collecting bottles beneath the cages during convulsive seizures. Such spillage would dilute the urine, causing an apparent low specific gravity. In the present study such an experimental error was impossible and no changes in kidney function could be detected.

Hematologic and biochemical studies similar to those reported here have been conducted by Newell *et al.* (7), whose results parallel those of the present report in all instances in which similar determinations were done. In addition to the extensive negative findings reported, these workers found that during periods of intoxication there was a definite increase in both magnesium and potassium in the serum. Administration of these ions during periods of acute poisoning, however, failed to produce any intensification of symptoms. No explanation of the retention of these elements was offered by Newell *et al.* (7).

The alterations which occurred in the circulating acetylcholine and cholinesterase are the only biochemical evidence of toxic effects to be revealed by the present study. Although the changes were definite and statistically significant, the question of the specificity of such damages in this type of intoxication cannot be presently answered. Changes in the level of circulating cholinesterase are known to occur in relation to several disease processes or stress. For instance, cholinesterase is usually lowered in chronic liver disease, in hypogonadism and in the 'alarm reaction' (8-10). In the present study there was no demonstrable liver damage to explain the results. It may well be that the changes found were secondary to the convulsions, since only after the clinical signs were well advanced did the increase in acetylcholine and decrease in cholinesterase become pronounced. Nevertheless, it is of interest, in the light of their well known rôles in neuromuscular transmission, that acetylcholine and cholinesterase should undergo such changes in the presence of an unknown neurotoxin, whose chief effect in dogs is the production of convulsions.

At this writing the only biochemical changes which have been described in agene intoxication involve the blood magnesium and potassium and the acetylcholine-cholinesterase relationship described in the present communication. It follows that the toxic signs may be based upon changes of a subtle nature, possibly within a cerebral enzyme system.

## SUMMARY

The metabolism of 6 dogs was studied intensively to determine what, if any, changes occur in conjunction with intoxication from the ingestion of agenzized white wheat flour. There was a large increase in serum acetylcholine and a striking decrease in serum cholinesterase activity during intoxication. These changes were progressive, being greatest toward the end of the period of intoxication. No impairment of kidney or liver function could be detected at any time, as judged by widely used clinical and laboratory criteria.

No consistent or biologically significant changes during the course of intoxication were detected in erythrocyte count, leukocyte count, blood hemoglobin, hematocrit, blood glucose, serum non-protein nitrogen, serum total protein, serum albumin, serum globulin, serum A/G ratio, serum urea nitrogen, serum inorganic phosphorus, serum calcium, urinary total nitrogen, urinary urea nitrogen, urinary creatinine, urinary calcium or urinary phosphorus.

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# ENZYME STUDIES ON HUMAN BLOOD. V. ESTIMATION OF PROTHROMBIN BY AN HOMOLOGOUS-ISOLATION METHOD<sup>1</sup>

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THIS laboratory has directed its investigations in the coagulation problem towards the thrombin-fibrinogen reaction. Thus, the effects of fibrinogen concentration and purity (1), of plasma proteins (2), and of heparin (3) on this reaction have been reported. In order that similar and other studies could be extended to the activation phase, it became apparent that a new technic for the determination of prothrombin must be developed since the one-stage (4, 5) and the two-stage (6, 7) technics did not fulfill certain requirements which included the following: homologous reagents; removal of fibrinogen without added thrombin; absence of acacia and albumin in both the activation and final reaction mixtures; a stable prothrombin fraction; and a system which could be applied in investigations of known and unknown coagulation dynamics and factors, other than prothrombin.

Reported in this communication is an homologous-isolation method for the determination of prothrombin. Fibrinogen is first removed as Fraction I by the application of the low temperature-alcohol principle of Cohn *et al.* (8) to a small volume of plasma. The supernatant fluid is then treated by a procedure similar to Mellanby's method (9) for the preparation of prothrombin concentrates except that native fibrinogen has been previously separated out. The prothrombin fraction is activated with calcium and placental thromboplastin with and without the presence of dilute fresh pooled normal plasma. The resulting thrombin is titrated with fibrinogen. Both the thromboplastin and fibrinogen reagents are of human origin and are quantitatively standardized. The final reference of potency is a thrombin preparation of the National Institutes of Health.

One unit of thrombin is defined here as that in 0.2 cc. volume which will clot 0.8 cc. of fibrinogen solution containing 0.15 to 0.3 per cent clottable protein in 15 seconds at  $\Gamma/2$  0.129, pH 7.2. One unit prothrombin is that which when fully activated at pH 7.2 25 to 28° C., with optimum concentration of calcium and known excess thromboplastin, will result in one unit of thrombin within 30 minutes. One unit of thromboplastin is the minimum required to convert one prothrombin unit to one thrombin unit.

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<sup>1</sup> Fibrinogen fractions were prepared in this laboratory from dried plasma processed from volunteer donors enrolled by the American Red Cross. Standard human thrombin powder was obtained through the courtesy of the Division of Biologic Control of the National Institutes of Health, Bethesda 14, Md.

The results by this new technic on 24 normal plasma samples are compared with those obtained by the thrombin method, a modification of the two-stage technic (7): Plasma is first defibrinated with thrombin; however, acacia has been omitted and homologous reagents are employed. Data on duplicate experiments with 7 other samples of plasma to evaluate the error of a single determination and of prothrombin recovery are also presented. In addition, methods for the preparation and standardization of human placental thromboplastin are described.

#### METHODS AND MATERIALS

*Citrate-Phosphate Buffer.* This buffer,  $\Gamma/2$ , 0.129 and  $pH$  7.2, consists of 250 cc.  $1/15$  M Sorensen buffer,  $pH$  7.1, and 75 cc. of 0.1 M sodium citrate in one liter solution (1) and unless otherwise indicated it was employed throughout this work. A glass electrode electrometer, standardized daily, was employed for all  $pH$  determinations.

*Buffered 53 Per Cent Ethanol.* Sufficient acetate buffer, 0.8 M,  $pH$  4.0, was added to 53.3 per cent ethanol (8) so that when 0.72 cc. of this buffered alcohol was mixed with 4 cc. of plasma at  $-3^{\circ}C.$ , the final  $pH$  would be  $7.2 \pm 0.2$ . In the present work on fresh liquid plasma, 0.8 cc. acetate buffer added to 72 cc. 53.3 per cent ethanol was adequate as evidenced by  $pH$  control experiments on separate aliquots of individual plasma samples. Similar studies should be made by each laboratory and for various types of plasma. This reagent was stored in a  $-25^{\circ}C.$  refrigerator.

*Preparation of Human Placental Thromboplastin.* Within two hours after parturition the placenta was stripped of the cord, large blood vessels and clots; rinsed with cold distilled water; sponged with gauze; and then weighed. When not in use, the glass bowl of the Waring Blendor was placed in a  $-25^{\circ}C.$  refrigerator. The temperature of the citrate-phosphate buffer and the centrifuge (International PR1) was between  $0^{\circ}$  and  $+1^{\circ}C.$  throughout the following procedure. The organ was cut into small slices, blended with an equivalent weight of citrate-phosphate buffer for 3 to 5 minutes, and then centrifuged at 700 g for 15 minutes. The sediment was again extracted with one-half volume of buffer. The two extracts were combined and the volume adjusted with buffer so that 150 cc. was equivalent to 100 gm. of tissue. This *crude tissue extract*, which was found to be stable indefinitely at  $-25^{\circ}C.$ , was then processed according to Chargaff's principle (10). A convenient volume, usually 150 cc., was centrifuged in an angle head at 32,000 g for 30 minutes. The supernatant fluid was decanted off and the sediment was blended approximately one-half minute with buffer, volume one-half that of the original crude tissue extract, and then centrifuged at 32,000 g for 30 minutes. This step was repeated 4 to 6 times or until the supernatant fluid contained less than 5 mg. per cent protein by the sulfosalicylic acid turbidity method (11). The final sediment was lyophilized, initial temperature was  $-25^{\circ}C.$  and pressure was 60 micra mercury. The resulting *crude thromboplastin powder* was weighed and stored over silica gel in a vacuum desiccator in which it was found to be stable indefinitely. *Stock thromboplastin solution* was prepared on the day of an experiment as follows: A 1 per cent suspension of the crude powder in citrate-phosphate buffer was stirred and heated at  $56^{\circ}C.$  for 5 minutes and then allowed to sediment for at least 15 minutes. The fluid was pipetted off and filtered through four layers of gauze.

*Standardization of the Thromboplastin Solution.* To 1 cc. of diluted pooled normal prothrombin fraction, 10 units/cc., was added 0.5 cc. of varying dilutions, 1 per cent to 100 per cent, of stock thromboplastin solution and 0.5 cc. 0.025 M  $\text{CaCl}_2$  and tested as in the isolation technic described below. The lowest concentration of the stock thromboplastin solution resulting in the maximum conversion of prothrombin to thrombin was considered the end point. Both the stock and working solutions did not clot upon addition of thrombin, did not clot fibrinogen solutions in the presence of optimum calcium concentration, and were stable for at least one working day at room temperature.

*Standardization of Calcium.* The optimum calcium concentration was determined by essentially the same procedure as employed for the thromboplastin standardization. To activation mixtures were added 0.5 cc. volumes of calcium chloride solutions, 0.005 to 0.100 M. That concentration, 0.025 M, resulting in maximum thrombin formation was selected as the standard reagent.

*Standard Fibrinogen Solution.* Fraction I, of at least 60 per cent purity in respect to fibrinogen, was prepared from human plasma by *method 6* of Cohn *et al.* (8). A 1 per cent suspension of the powder was stirred in citrate-phosphate buffer at room temperature for 20 minutes and then centrifuged for 20 minutes at 1500 g. The supernatant fluid, on which total and clottable protein was analyzed for each lot of Fraction I powder, was then diluted with buffer to 0.15 to 0.30 per cent fibrinogen, the optimum concentration as previously established (1). This standard solution did not clot at room temperature in one working day nor did the clotting time with standard thrombin vary significantly during this period.

*Standard Thrombin Solution.* Human thrombin from the National Institutes of Health (N.I.H.) 12.5 U/mg., was dissolved in citrate-phosphate buffer to contain 50 U/cc. Small aliquots were stored at  $-25^\circ\text{C}$ . The working solution, usually a 1 to 10 dilution of the stock, was stable for at least one day in an ordinary refrigerator.

*Titration of Thrombin.* The stop watch with a hidden face was started at the moment 0.2 cc. of thrombin solution or prothrombin activation mixture was blown into 0.8 cc. standard fibrinogen solution, contained in a test tube (i.d., 1 cm.) previously equilibrated in a  $37.5^\circ\text{C}$ . water bath for 20 seconds. During the first 4-second period, the pipette was put aside and the test tube was shaken. Then the tube was placed in an almost horizontal position, lowered to a  $25^\circ$  angle and restored to the horizontal position at the rate of 3 cycles every 5 seconds. The first visible clot formation was taken as the end point. The unit of thrombin/0.2 cc. was found by referring to a clotting time concentration curve (fig. 1) obtained by repeated tests with N.I.H. thrombin solutions. This reference curve should be prepared by each laboratory.

*Collection of Blood.* Ten volumes of blood was obtained from each normal individual with a syringe containing one volume of 4 per cent sodium citrate solution. The blood was centrifuged in calibrated 15 cc. graduated tubes at  $1^\circ\text{C}$ ., 1650 g, for 30 minutes. Therefore, the plasma hematocrit was read directly so that corrections could be made for the citrate dilution. For this particular study, 15 cc. citrated whole blood was obtained since prothrombin was determined by two methods and other

analytical tests were done. For the isolation technic alone, 10 cc. blood added to 1 cc. sodium citrate was found to be adequate.

*Isolation Technic for Prothrombin.* Exactly 4.0 cc. of fresh citrate plasma was pipetted into a test tube measuring 13 mm. (i.d.) by 85 mm. The tube was then placed in a  $-3^{\circ}\text{C}$ . alcohol bath. When the temperature of the plasma was  $0^{\circ}\text{C}$ ., 0.72 cc. buffered alcohol was added with constant mechanical stirring. A calibrated 1 cc. tuberculin syringe and a no. 24-gauge needle were used for the alcohol addition which took approximately one-half minute. The alcohol-plasma mixture was then stirred for at least 15 additional minutes and then centrifuged at  $-3^{\circ}\text{C}$ ., 1670 g, for 25 to

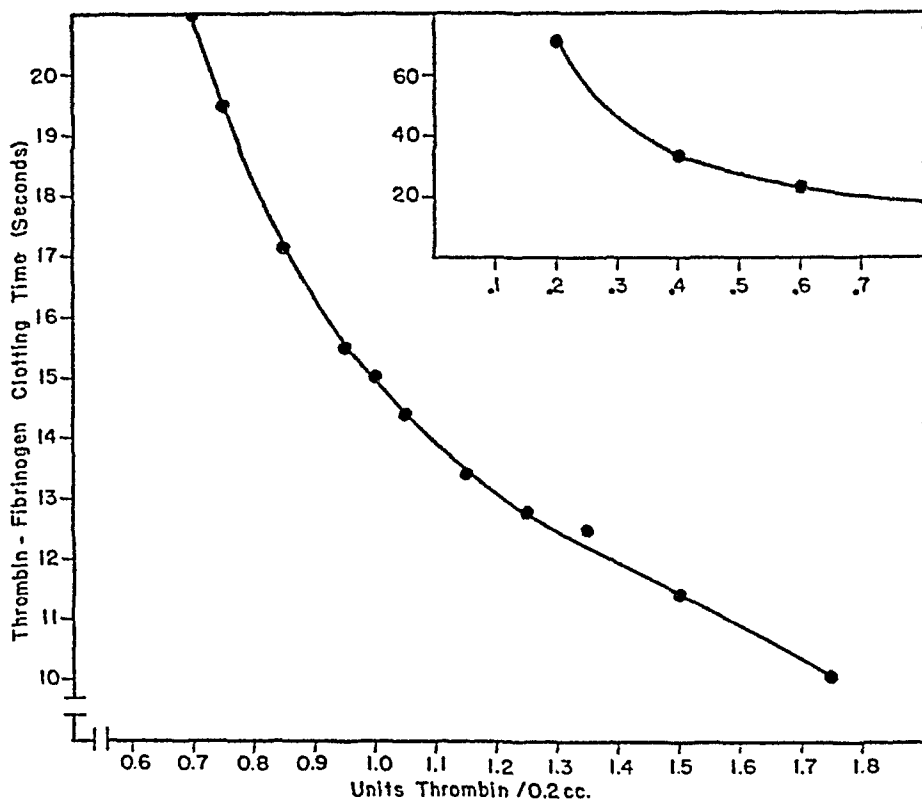


Fig. 1. CLOTTING TIME of human fibrinogen solution with varying concentrations of thrombin. Thrombin 0.2 cc. added to 0.8 cc. of 0.25 per cent fibrinogen solution previously equilibrated at  $37.5^{\circ}\text{C}$ . Citrate-phosphate buffer,  $\Gamma/2$ , 0.129;  $p\text{H}$  7.2.

30 minutes. The fibrinogen fraction precipitate, which was saved for other studies (12), was firmly packed. Therefore, the test tube could be completely inverted, resulting in an almost quantitative transfer of the supernatant fluid into 40 cc. of  $0^{\circ}\text{C}$ . distilled water in a 50 cc. centrifuge tube with constant mechanical stirring and with temperature maintained at  $0^{\circ}$  to  $+1^{\circ}\text{C}$ . Then, 2.8 cc. 0.10 M acetic acid was added to a  $p\text{H}$   $5.05 \pm 0.05$ . The turbid solution was stirred for an additional 5 minutes. In 20 to 30 minutes, the now flocculent solution was centrifuged at  $+1^{\circ}\text{C}$ ., 1670 g, for 30 minutes. The supernatant fluid was decanted off and the tube inverted for one minute at  $0^{\circ}$  to  $+1^{\circ}\text{C}$ . The inner wall of the tube was wiped with a clean chilled gauze without disturbing the precipitate. Immediately, 4 cc. of  $0^{\circ}\text{C}$ . citrate-phosphate buffer was added directly to the sediment and stirred with a 1 cc. sero-

logical pipette two or three times during the next 20 minutes. The resulting solution, pH 7.0-7.1, was stable for at least one day in an ordinary refrigerator and therefore no special haste to proceed to the next step was necessary. To 0.15 cc. or more of the prothrombin solution was added buffer to 1 cc. volume and 0.5 cc. thromboplastin solution, 50 U/cc. The addition of 0.5 cc. 0.025 M calcium chloride solution to the mixture at 25 to 28° C. began the activation time. Thrombin was determined on 0.2 cc. aliquots at least three times during the last 10 minutes of the 30-minute activation period. A mean of the two lowest consecutive clotting times was taken as the end point. An identical activation mixture, except that 0.2 cc. of 1 to 40 diluted fresh normal pooled plasma was substituted for an equal volume of buffer, was also made up and similarly titrated for thrombin. As control measures to make certain the absence of fibrinogen and thrombin in the prothrombin solution, 0.8 cc. and 0.2 cc. aliquots were added to 0.2 cc. (1 U) thrombin and 0.8 cc. standard fibrinogen respectively, and the clotting times taken. The prothrombin fraction remaining from each test was frozen at -25° C. or lyophilized and saved for other studies.

*Thrombin Technic for Prothrombin.* To 1 cc. of fresh citrated plasma was added 1 cc. (5 U) of thrombin. Clotting occurred in less than 15 seconds. In 5 to 10 minutes the clot was retracted and removed with an applicator stick. Without delay 0.25 cc. or more of the defibrinated plasma was activated as in the isolation technic with the following differences: A more concentrated thromboplastin solution (500 U/cc.) was necessary and the thrombin titrations were done every minute beginning two minutes after the calcium addition. The mean of the two lowest consecutive clotting times was taken as the end point. For a control, 0.2 cc. of the defibrinated plasma was added to 0.8 cc. of standard fibrinogen solution to test for possible thrombin. In every test the activation mixture was carefully examined for the presence of fibrin granules or clots.

*Calculation of Prothrombin.* The following equation was employed:  $T \times \frac{1.0 \text{ cc.}}{0.2 \text{ cc.}} \times \frac{2.0 \text{ cc.}}{V} \times C = \text{U/cc. plasma}$ ; where  $T$  was the units thrombin obtained from figure 1;  $C$  was the correction for the citrate dilution of plasma; and, in respect to the activation mixture, 0.2 cc. was the volume titrated for thrombin,  $V$  was the volume in cc. of prothrombin solution or defibrinated plasma added, and 2.0 cc. was the total volume. Therefore, in the isolation technic the above equation was simplified:  $T \times \frac{10}{V} \times C = \text{U/cc. plasma}$ ; and in the thrombin technic, since the plasma was diluted initially with an equal volume of thrombin,  $T \times \frac{20}{V} \times C = \text{U/cc. plasma}$ .

## RESULTS

The clotting time data from several experiments with human Fraction I and National Institutes of Health thrombin, 12.5 U/mg., are depicted in figure 1. The resulting curve between 12 and 18 seconds generally constituted the thrombin titration standard for this study. Clotting times outside of this range were utilized for exceedingly low concentrations of prothrombin, below 10 U/cc. by the isolation

technic, and for the estimation of the most suitable volume of the solution to be added in the activation mixture.

Another series of experiments was made to demonstrate the effect of acacia on the titration of thrombin. The stock 15 per cent acacia solution was prepared according to Ware and Seegers (7). In each experiment, N.I.H. thrombin was weighed and dissolved in citrate-phosphate buffer to contain 10 U/cc. The results in table 1 show that acacia greatly accelerates the thrombin-fibrinogen reaction time and indicates that one unit as determined in this laboratory equals 1.0 N.I.H. unit or 2.46 units of Ware and Seegers (7) when human fibrinogen fraction prepared by Cohn's method (8) was used as the substrate.

Figure 2 demonstrates not only the results obtained by varying concentrations of thromboplastin but also the greater requirements of this reagent by thrombin-defibrinated plasma than by prothrombin fractions. Therefore, since a 4 per cent stock solution was the minimum concentration resulting in the lowest clotting time

TABLE 1. EFFECTS OF ACACIA AND OTHER FACTORS ON TITRATION OF THROMBIN

COMPONENTS: TITRATION MIXTURE				RESULTS			
Thrombin Solution		Human Fraction I		Units Found/Theory <sup>1</sup>			
Acacia Conc.	Vol.	Conc.	Vol.	Experiment			Mean
<i>per cent</i>	<i>cc.</i>	<i>%</i>	<i>cc.</i>	<i>A</i>	<i>B</i>	<i>C</i>	
0.0	0.2	0.3	0.8	0.99	1.06	0.96	1.00
0.0	0.4	1.0	0.1	0.92	1.07	0.97	0.99
2.5	0.4	1.0	0.1		2.52	2.39	2.46

<sup>1</sup> Three N.I.H. thrombin solutions containing 10 U/cc. were diluted so that clotting times ranged between 13 and 17 seconds on the thrombin-clotting time curve (fig. 1)

a 2.5 times stronger solution or a 10 per cent stock solution was employed in the isolation technic. Similarly, 2.5 × 40 per cent or a 100 per cent stock thromboplastin solution was the working reagent used in the thrombin technic.

The foregoing results are identical to those of many other experiments. The average yield of the crude thromboplastin powder was 580 mg/100 gm. human placental tissue. The average potency results from 5 experiments in units thromboplastin expressed variously were as follows: 29,000/100 gm. tissue; 50/mg. crude powder; 500/cc. stock solution; or 5000/mg. total tyrosine equivalent.

A typical experiment showing the widely diverse activation curves obtained by the thrombin and the isolation technics is depicted in figure 3. In the former, there was a critical minimal clotting time, between 5.5 and 8.5 minutes after the addition of calcium in plasma samples with normal prothrombin. With lower concentrations of prothrombin, the optimum activation time occurred more rapidly and the anti thrombin activity was even more apparent. On the other hand with the isolation technic and with all ranges of prothrombin, the clotting time dropped up to 20 minutes and then levelled off during the remainder of the 30-minute activation period.

In the experiments with the isolation technic the total tyrosine equivalents of

the whole citrated plasma and its various fractions were determined with phenol reagent (1). The Coleman Junior Spectrophotometer at  $650\text{ m}\mu$  was the instrument employed. The results in table 2 clearly demonstrate chemically the efficiency of the fractionation procedure. The mean sum of the tyrosine equivalents of all the fractions was 95.6 per cent of that found in whole plasma. The prothrombin fraction represented approximately 5.5 per cent of the total tyrosine equivalent of the original sample.

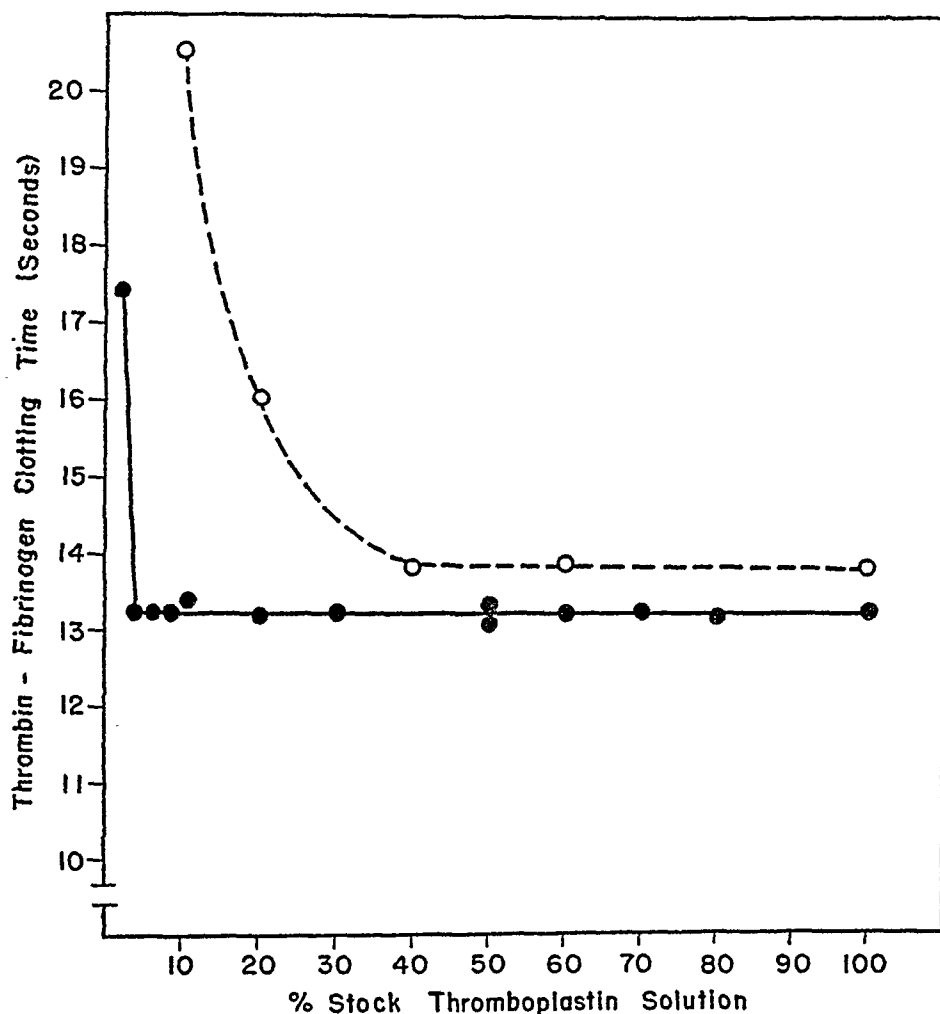


Fig. 2. VARIATION IN THROMBOPLASTIN REQUIREMENTS in two technics.  $\bigcirc$  - -  $\bigcirc$ : Thrombin technic; optimum activation time, 5.5-8.5 minutes.  $\bullet$  - -  $\bullet$ : Isolation technic; optimum activation time, 20-30 minutes. Activation:  $25$ - $28^{\circ}\text{C}$ .;  $\text{pH}$  7.2.

Another series of experiments, in duplicate, were done on 7 individual samples of plasma to evaluate the error of a single determination at varying concentrations of prothrombin. In each of plasma no. V, VI and VII, the experimental procedure was as follows: Twice-Seitz filtered plasma (20 cc/6 cm. S.T. pad) was mixed with varying volumes of the original plasma. The results by both technics are recorded in table 3. It can be concluded from the analysis of variance on data obtained on a series of duplicate tests, zero prothrombin results excluded, that the error of a single determination in the isolation technic was 2.1 U/cc. (11 degrees of freedom) in plasma

samples with prothrombin ranging between 20 and 85  $\text{U/cc}$ ; and in the thrombin technic, 4.8  $\text{U/cc}$ . (10 degrees of freedom) between 20 and 100  $\text{U/cc}$ . That plasma treated simply by twice-Seitz filtration, for all practical purposes, does not contain prothrombin was determined as follows: Prothrombin fractions were obtained by the isolation technic. Maximum volumes, 0.8 cc. of this fraction, were added in the activation mixtures. After 20, 30 and 40 minutes activation, 0.2 cc. volumes of the mixtures were added to 0.8 cc. standard fibrinogen solutions. Clots and granules

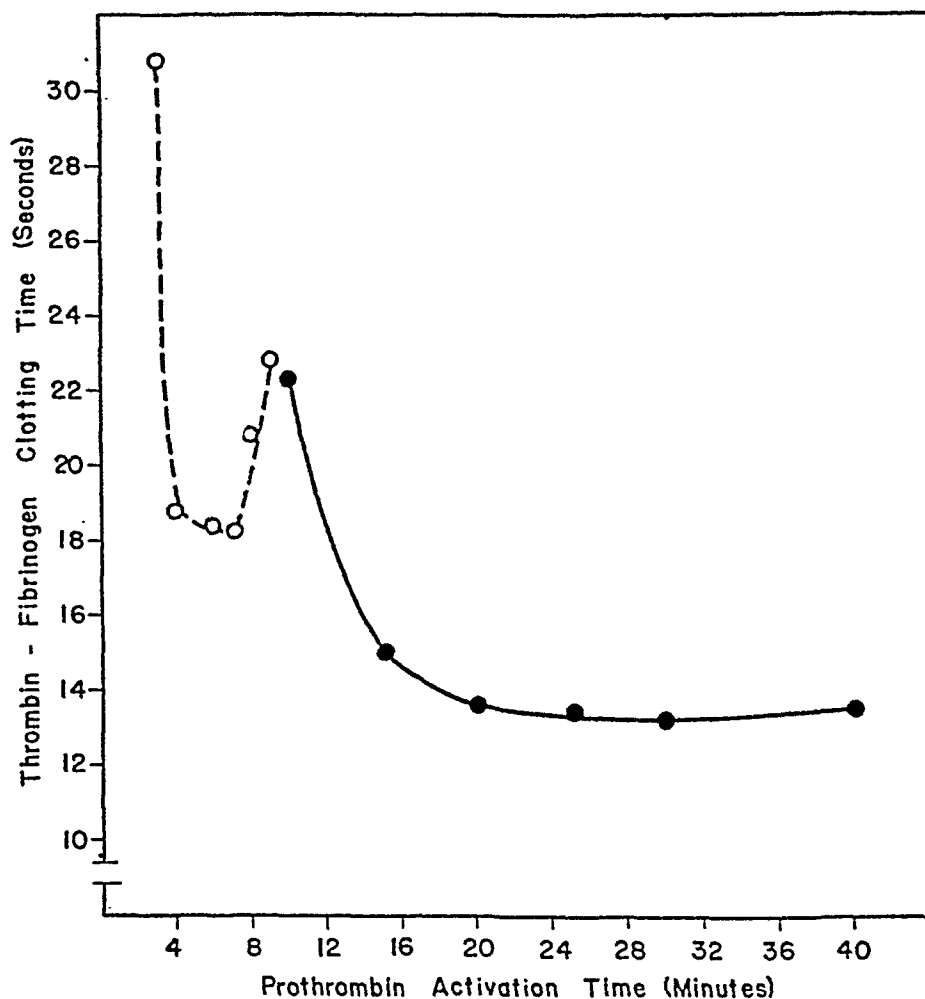


Fig. 3 VARIATIONS IN ACTIVATION PHASE of two technics.  $\bigcirc$  --  $\bigcirc$ : Thrombin technic; 0.20 cc. defibrinated plasma in activation mixture.  $\bullet$  —  $\bullet$ : Isolation technic; 0.20 cc. prothrombin fraction in activation mixture. Activation: 25–28° C.; pH 7.2.

were absent after 70 seconds, 4 hours, and even 60 hours of observation. In identical experiments with the same volume of prothrombin fractions containing, however, 2.5  $\text{U/cc}$ ., definite clots formed in 70 seconds in contrast to no clot in 60 hours. In experiments with mixtures of filtered and non-filtered plasma, the prothrombin data in table 3 calculated for the found/expected percentages were as follows: isolation technic, 96.2, 92.2, 106.0 and 99.5 per cent; thrombin technic, 80.9, 114.0 and 84.0 per cent. The foregoing results obtained with the isolation technic were inter-

preted as additional evidence for the complete removal of prothrombin with Seitz filtration alone.

The mean prothrombin content in U/cc. of fresh plasma on 24 normal individuals ranging in age from 21 to 34 years are recorded in table 4. The correlation between the data obtained by the two technics indicated only that the results were generally lower with the isolation technic. The thrombin controls in all tests by both technics were negative in 2 minutes and in 24 hours. In the isolation technic, the fibrinogen controls showed no clots or granules after 2 minutes, when one unit of thrombin was added to 0.8 cc. of prothrombin fraction. That fibrinogen was completely removed in the defibrinated plasma was evident in the thrombin technic by the absence of fibrin clots or granules in the activation mixture. The addition of fresh normal pooled human plasma in 1 to 400 concentration in the activation mixtures did not have any effect on the results by either technic.

TABLE 2. FRACTIONATION OF PLASMA BY THE ISOLATION TECHNIC

WHOLE PLASMA OR FRACTIONS		N	TOTAL TYROSINE EQUIVALENT/CC. PLASMA		
			Mean mg.	$\pm$ S.D. mg.	C.V. %
Whole Plasma		29	3.010	.280	9.14
Fractions	I	24	.311	.051	16.40
	Prothrombin	24	.167	.017	10.18
	pH 5.05 Supernatant	24	2.400	.220	9.10
	Total	24	2.878		

## DISCUSSION

Any attempt to interpret the results obtained in this study requires first a critical examination of the analytical technics employed as compared with other methods for the estimation of prothrombin. The similarity of the thrombin technic and the two-stage method of Warner, Brinkhous and Smith (6) as further modified by Ware and Seegers (7) is evident in table 4. The normal result for prothrombin in fresh human plasma is reported by the latter as approximately 172 U/cc. with bovine Fraction I as the substrate in the thrombin titration; and 300 to 350 U/cc. with a more reactive fibrinogen preparation. In the present work, 110.0 U/cc. was found with the thrombin technic which differs principally in that standard fibrinogen fraction and thromboplastin of human instead of bovine origin are used and that acacia is absent. Acacia is employed in the modified two-stage technic to permit a greater dilution of plasma resulting thereby in the diminution of antithrombin activity (7). However, it was omitted here, since in all our studies on factors influencing prothrombin conversion only physiological reagents have been used. It was found that acacia alone causes a 2.46 times greater reactivity in the thrombin-fibrinogen reaction, confirming the report of Seegers and Smith (13). Therefore,

acacia is, at least, a partial explanation for the higher values for fresh normal human plasma with the modified two-stage technic of Ware and Seegers (7) than with the similar thrombin technic.

The results on the same specimens with the isolation technic averaged 82.4 U/cc. which is somewhat lower than that obtained by the thrombin technic. There are several other concrete differences in the two methods. Defibrination with thrombin is not necessary in the isolation technic, since native fibrinogen is separated out as Fraction I. It may be more appropriate to designate the thrombin technic as a three-stage procedure since the reaction of added thrombin with plasma constitutes an active coagulation process. Ware and Seegers (14) found that thrombin has variable effects on prothrombin, depending on their relative concentrations.

TABLE 3. DUPLICATE ANALYSIS BY THE ISOLATION AND THROMBIN TECHNIQUES ON SAMPLES CONTAINING VARYING CONCENTRATIONS OF SEITZ-FILTERED PLASMA

% SEITZ-FILTERED PLASMA IN SAMPLE	PROTHROMBIN RESULTS: U/CC. PLASMA							
	Isolation Technic				Thrombin Technic			
	a	b	Mean	Expected	a	b	Mean	Expected
0% I	49.6	51.2	50.40		72.0	71.6	71.80	
0% II	57.3	60.8	59.05		73.6	80.4	77.00	
0% III	60.5	63.2	61.85		85.4	95.6	90.50	
0% IV	58.6	55.6	57.10		83.7	88.0	85.85	
0% V	63.9	68.3	66.10		90.4	96.0	93.20	
50% V	32.3	31.7	32.00	33.05	36.6	38.8	37.70	46.60
100% V	0.0	0.0	0.00		0.0	0.0	0.00	
0% VI	69.3	70.0	69.65		89.6	104.0	96.8	
50% VI	33.3	30.7	32.00	34.83				
100% VI	0.0	0.0	0.00					
0% VII	87.5	82.1	84.80		99.7	95.0	97.35	
50% VII	46.6	43.3	44.95	42.40	54.3	54.7	55.50	48.68
75% VII	21.2	21.0	21.10	21.20	23.1	17.8	20.45	24.34
100% VII	0.0	0.0	0.00		0.0	0.0	0.00	

Another difference in the two methods lies in the nature of the prothrombin conversion. It has been shown in this study that the maximum thrombin titer in human plasma with the thrombin technic occurs at a critical point after which the enzyme rapidly disappears. This phenomenon has been previously demonstrated by Warner, Brinkhous and Smith (6). Even with the modified two-stage technic, Ware and Seegers (7) have reported the problems arising from increasing antithrombin activity in low dilutions of plasma and therefore in low prothrombin values. On the contrary, with the isolation technic, the thrombin titer reaches a maximum in approximately 20 minutes and then it is stable for the remainder of the 30-minute activation period and longer. Evidently, substances acting as antithrombin have been largely removed in this technic.

In another communication (2) it was shown that plasma proteins have a pronounced effect on the thrombin-fibrinogen reaction. However, in the isolation technic,

the low protein concentration (see table 4) would exclude this effect as an accessory factor.

The thromboplastin requirement in the thrombin technic is approximately 10

TABLE 4. CHARACTERISTICS OF FOUR TECHNIQS FOR DETERMINATION OF PROTHROMBIN

CHARACTERISTICS	ISOLATION PRESENT STUDY	THROMBIN PRESENT STUDY	MODIFIED TWO-STAGE WARE-SEEGERS (7)	ONE-STAGE QUICK (4, 5)
Removal: Plasma Fibrinogen	As Fraction I	With Thrombin	With Thrombin	None
Activation mixture:				
Calcium conc. M	0.0062	0.0062	0.0039	0.0083
Thromboplastin source	Human placenta	Human placenta	Bovine lung	Rabbit brain
Thromboplastin conc.	12.5 U/cc.	125.0 U/cc.	excess	excess
Albumin <sup>1</sup> % conc.	0.000	0.180	0.021	1.000
Globulin <sup>1</sup> % conc.	0.024	0.180	0.021	1.000
Fibrinogen <sup>1</sup> % conc.	0.000	0.000	0.000	0.100
Acacia % conc.	0.000	0.000	2.500	0.000
Labile factor (s) added	Human plasma	Human plasma	Bovine serum	None
Titration mixture:				
Fibrinogen source	Human Fraction I	Human Fraction I	Bovine Fraction I	Same as activation mixture
Fibrinogen % conc.	0.12-0.24	0.12-0.24	app. 0.2	
Albumin <sup>1</sup> % conc.	0.000	0.036	0.017	
Globulin <sup>1</sup> % conc.	0.005	0.036	0.017	
Acacia % conc.	0.000	0.000	2.000	
Standard	Human thrombin	Human thrombin	Thrombin	12.5" = 100%
Normal Values				
N	24	24	5 <sup>2</sup>	
Mean	82.4 U/cc.	110.0 U/cc.	172.0 U/cc.	> 70% of normal
±S.D.	17.3 U/cc.	14.6 U/cc.		

<sup>1</sup> Based on human plasma with 3% albumin, 3% globulin, 0.3% fibrinogen, normal prothrombin.

<sup>2</sup> Dr. Walter H. Seegers, Wayne University, Detroit. Personal communication, 1949.

times greater than that in the isolation technic. It is concluded that, in the latter, any antithromboplastin activity has been largely eliminated. Experience during the last 10 years in this laboratory has shown that the standardization of thrombo-

plastin reagent is the most important factor influencing the accuracy of prothrombin determinations with any technic. Therefore, in the present study, this reagent is titrated with prothrombin solutions of known activity and a 2.5 times excess employed in the two technics. The latter was arbitrarily chosen as sufficient for an adequate margin of safety in analysis of normal plasma: It is also an application of the concept of a controlled excess of a component in the study of a biological system. The simple procedure described here resulted in human thromboplastin preparations which satisfied the criteria established for the present study. These included stability, standardization, high yield, moderate activity and, most important, absence of other coagulation factors. The last was achieved by repeated high gravity centrifugation and by heating at 56° C., and confirmed by control tests. The ready availability of fresh human placenta in comparison to brain or lung of any species requires no further comment.

One of the most important of recent developments in the field of coagulation has been the discovery of the new Factor V of Owren (15), accelerator factor of Fantl and Nance (16), Ac-globulin of Ware, Guest and Seegers (17), and the labile or plasmatic co-factor of Quick (18) and Honoratio (19). This factor was considered in this study by the introduction of fresh diluted plasma into prothrombin conversion mixtures. However, no significant change in the conversion rate or the strength of prothrombin could be observed over the controls in any of the determinations in fresh normal plasma by either the isolation or thrombin technics. Therefore, it is assumed that this new factor existed in adequate amounts. Murphy and Seegers (20) have shown that fresh normal human plasma contains very little Ac-globulin and that almost identical prothrombin results are obtained with or without the added factor.

#### SUMMARY

An homologous isolation technic for the determination of prothrombin on 4 cc. samples of plasma is described. It is based on the low temperature, low ionic strength alcohol removal of Fraction I (Cohn) and the subsequent isoelectric precipitation of a stable thrombin- and fibrinogen-free prothrombin fraction. The latter is activated with an optimum concentration of calcium and a known excess of placental thromboplastin. The resulting thrombin is titrated with a fibrinogen solution. A standard thrombin preparation is the final reference of potency. Methods for the preparation and standardization of fibrinogen and thromboplastin are outlined. Results from experiments to evaluate the error of a single determination, the recovery of prothrombin, the efficiency of the fractionation, and the reaction with thromboplastin are presented. The average value obtained on 24 normal individuals ranging in age from 21 to 34 years is 82.4 U prothrombin/cc. plasma.

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# EFFECT OF BLOOD PLATELETS ON PROTHROMBIN UTILIZATION OF DOG AND HUMAN PLASMAS<sup>1</sup>

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IT HAS been demonstrated, qualitatively, that the rate of blood coagulation is related to the presence of blood platelets. This relationship has been known to exist in bird blood since the investigations of Delezenne (1). In mammalian blood, however, conflicting data and opinions regarding the rôle of platelets in coagulation have been published during the past half-century. Only in the past decade has it been clearly demonstrated that platelets play an important rôle in the clotting process. In 1939 one of us (2) showed that prothrombin is slowly utilized in human blood if it is immediately centrifuged to reduce the number of platelets present during clotting. More recently, Jaques and co-workers (3) showed that platelet-poor plasmas have a prolonged clotting time. In their experiments, platelet alterations during the preparation of plasma were prevented by use of silicone-treated equipment. Later work demonstrated that plasma, carefully prepared with a silicone technique to minimize or prevent platelet rupture, clots very slowly or not at all when freed of platelets. Incoagulable platelet-free plasma has been obtained from both human and dog blood (4, 5).

The purpose of this investigation was to determine the platelet levels at which impairment of the clotting process appears, and to compare the platelet requirements of human and dog blood.

## METHODS

The method for obtaining the blood and plasma was as follows: syringes, needles (16-18 gauge) and glassware were treated with a 10 per cent solution of a methychlorosilane (General Electric Dri-Film) in benzene. Blood was obtained from the external jugular vein in the dog and from the cephalic vein in the human. No samples were used in which difficulties were encountered in the venepuncture or in blood withdrawal, or in which there was evidence of lipemia. The blood was collected in a series of three syringes. In the first syringe, 4 to 6 ml. of blood were obtained for determination of the hematocrit. In the second syringe, 4 ml. of blood were obtained for platelet counts. And in the third, 45 to 50 ml. of blood were obtained without anticoagulant for the clotting studies. Blood from the last syringe was cooled rapidly in silicone-treated tubes in an ice bath. To reduce the number of platelets to the desired level, the blood was centrifuged for varying periods of time in angle centrifuges. For dog samples, a centrifugal force of about 1450 g for 2 to 15 minutes was used. For human samples the centrifugal force was about 1650 g for the same periods of time. To obtain platelet-poor plasmas containing less than 5,000 to 10,000 platelets per cu. mm., a centrifugal force of about 15,000 g was used. Collection and centrifugation of the blood were carried out in a constant temperature room (2° C.).

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Two measures of the rate of clotting were used, the clotting time and the prothrombin utilization rate. In both tests, timing of the clotting process was started when the blood or plasma was placed in ordinary glass tubes. For the clotting time determinations, 1 ml. of whole blood or 0.5 ml. of native plasma was transferred with a silicone-treated needle and syringe to each of two dry 10 x 75 mm. glass tubes. For the determination of the rate of prothrombin utilization (2), blood or plasma was transferred in a similar manner to a series of tubes containing 0.15 ml. of imidazole buffer at pH 7.3 (6). At frequent intervals during the next 50 to 60 minutes, the contents of each of two tubes were mixed with 0.12 to 0.16 ml. of 3.2 per cent sodium citrate solution to stop the conversion of prothrombin to thrombin. Prothrombin determinations, using the two-stage method of Warner, Brinkhous and Smith (7, 8), were made promptly on the plasma or serum. The above procedures were carried out at 27° to 28° C.

Platelet counts on the whole blood were performed by a modification of Nygaard's method (9), using 4 parts of 3.2 per cent sodium citrate to one part of whole blood. After sedimentation of dog blood for 15 minutes, and of human blood for 30 minutes, supernatant plasma was transferred to a counting chamber and the platelets counted. Direct platelet counts were made on the native

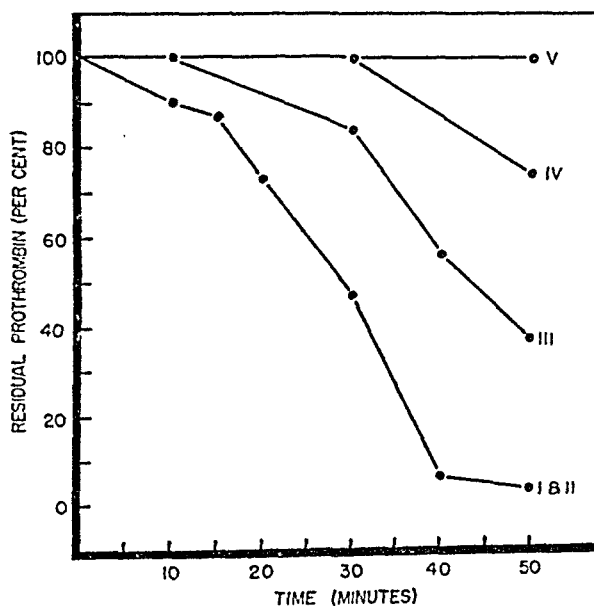


Fig. 1

plasma specimens obtained by centrifugation. One of us (J. A. B.) performed all the counts, which were done in duplicate or triplicate.

## RESULTS

Sixteen healthy adult dogs and 9 healthy adult human subjects, selected without regard to sex, were used. In all, 48 experiments were performed on dog blood and 32 experiments on human blood. The mean number of platelets for dogs was 383,000/cu. mm. of whole blood, or 658,000/cu. mm. of plasma, with a standard deviation of 82,000. The mean value for the human subjects was 285,000/cu. mm. of whole blood, or 549,000/cu. mm. of plasma, with a standard deviation of 59,000.

The results of one group of experiments with dog blood are given in figure 1 and table 1. This group exemplifies the experimental procedure followed throughout this work. The residual prothrombin content of the whole blood and plasma samples during the course of clotting is shown in the figure. It is seen that in the plasma with 58 per cent of the original number of platelets (curve II), the prothrombin disappeared

at the same rate as in the whole blood (curve I). Plasmas containing 22 per cent of the original number of platelets or less (curves III and IV) showed a considerable retardation of the rate at which prothrombin disappeared. The plasma in which only a few platelets remained showed no loss of prothrombin during the experimental period (curve V).

To obtain a numerical expression of the extent of the retardation of clotting, a prothrombin utilization index was devised. This index represents the ratio of the amount of prothrombin utilized in the plasma to the amount utilized in the normal whole blood. The method of calculation of the index is shown in table 1. The interpolated points on the whole blood curve (fig. 1) at which 75, 50, and 25 per cent of the original prothrombin remained in the serum were selected. The times on the

TABLE 1. EFFECT OF PLATELET CONTENT OF PLASMA ON CLOTTING TIME AND PROTHROMBIN UTILIZATION INDEX

CURVE NO. (see fig. 1)	SPECIMEN	PLATELETS		CLOTTING TIME	PROTHROMBIN UTILIZED			PROTHROMBIN UTILIZATION INDEX
		Per cu. mm. of plasma $\times 10^4$	No. relative to whole blood		Minutes			
					19	28	35	
I	Whole blood	650	%	min.	%	%	%	
II	Plasma (2 min. centrifugation)	380	100	9	25	50	75	$\frac{150}{150}$ or 1.0
III	Plasma (3 min. centrifugation)	140	58	10	25	50	75	$\frac{54}{150}$ or 0.36
IV	Plasma (4½ min. centrifugation)	86	22	14	8	15	31	$\frac{8}{150}$ or 0.05
V	Plasma (150 min. centrifugation)	<0.3	13	27	0	0	8	$\frac{0}{150}$ or 0.00
			<1	>35	0	0	0	$\frac{0}{150}$ or 0.00

abscissa were found to be 19, 28, and 35 minutes respectively. Then the corresponding prothrombin values on the plasma curves were obtained. The average ratio of the prothrombin *utilized* at the indicated times was then determined. Values of less than 1.0 indicate that clotting is impaired—the slower the clotting the lower the value of the prothrombin utilization index.

Figures 2 and 3 show the relationship between the platelet content of plasma and the rate of clotting. In the dog plasmas, it will be observed that an impairment of clotting occurred when the number of platelets was reduced below about 35 per cent of the number present in whole blood, or about 230,000 platelets per cu. mm. of plasma (fig. 2). In the human plasmas, on the other hand, no impairment of clotting was evident until the number of platelets was below about 25 per cent of the original value, or about 135,000 platelets per cu. mm. of plasma (fig. 3). When the platelets were reduced to a range of 5 to 15 per cent in dog plasma, frequently no prothrombin disappeared during the period of observation. In these cases, the prothrombin utilization index was 0. In human plasma, considerably greater reduction in the number of

platelets was required to prevent utilization of prothrombin in the experimental period.

The clotting time was a less sensitive index of changes in the course of clotting than was the prothrombin utilization rate. In human plasma, even with a reduction of platelet levels to 5 per cent of the original whole blood values, no prolongation of the clotting time was noted. Only when the platelets were at a level of about one per cent or lower, was a consistent delay in clotting time observed. In dog plasma, on the other hand, a prolongation of the clotting time was observed regularly when the platelet levels were reduced below about 15 per cent of the original values. With extremely low platelet counts in either type of plasmas, clotting generally did not occur during the 50 to 60 minute period of observation.

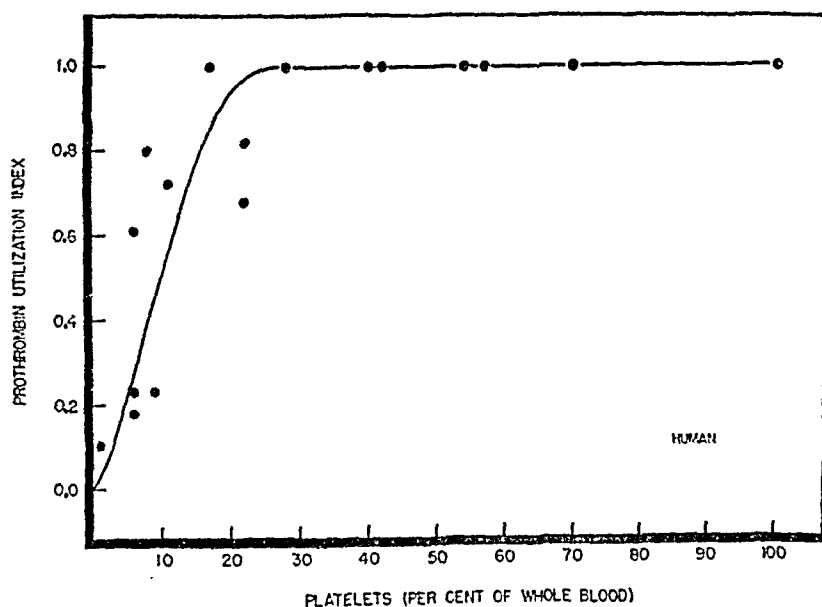


Fig. 2

#### DISCUSSION

These results emphasize the need for platelets in clotting and indicate that normally platelets are present in numbers considerably in excess of minimal requirements for normal clotting. Roughly, there is a three-fold factor of safety in dog blood, and a four- or five-fold safety factor in human blood. The difference between the platelet needs of dog and human blood is more striking if considered in terms of platelet volume. Normally, according to Van Allen (10), the average volume percentage of platelets for dog blood is 1.04, for human blood 0.49. Thus, the minimal platelet volumes required for normal clotting would be about 0.36 ml/100 ml. of dog blood and about 0.12 ml/100 ml. of human blood. By volume, then, dog blood requires about three times as much platelet material as human blood, compared to about 1.7 times as many if considered in terms of platelet numbers.

The fundamental reason for the greater need for platelets in dog blood is not clear. Whether there are qualitative or quantitative differences in the platelet coagulant factors in the two species is not known. Earlier work has shown that the

plasma factor deficient in hemophilic blood is necessary for platelet utilization (4). It may be that the quantity of the anti-hemophilic principle in plasma determines the extent to which platelets are utilized in the clotting process. At any rate, our data indicate that normally the number of platelets is not the factor which determines the rate of coagulation, and only when their numbers are greatly reduced do they limit the speed of clotting.

A comparison of our findings on normal human plasma with results obtained on blood from patients with thrombocytopenia is of interest. In this disease, the clotting time is nearly always normal. Our data are in accord with this fact, since platelets rarely reach the low levels that would be required for a prolonged clotting time. Soulier (11) has used a modified two-stage method for the determination of residual prothrombin in the serum of a group of thrombocytopenic patients. Seven-

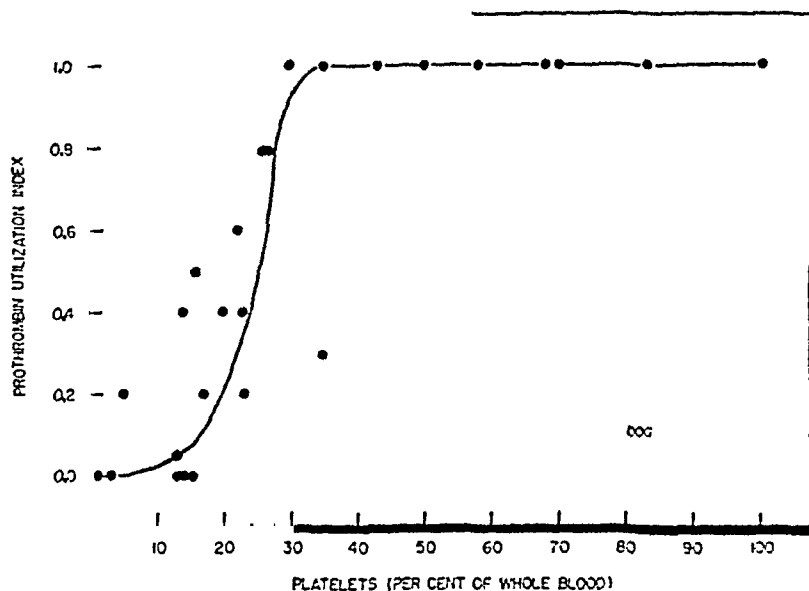


Fig. 3

teen of his patients showed a retardation in the clotting process, as judged by high serum prothrombin levels. All of these patients had platelet counts below 81,000/cu. mm. This is in good agreement with our data, which indicate that the average critical level of platelets is equivalent to a value of about 71,000/cu. mm. of whole blood. On the other hand, there is less evident agreement between our data and those of Conley, Hartmann and Morse (12) and of Quick, Shanberge and Stefanini (13). These authors studied both blood from thrombocytopenia patients and platelet-poor normal plasmas. From their work, it would appear that only a moderate reduction in the number of platelets is sufficient to cause a delay in clotting. They used a one-stage technique for determination of serum prothrombin. This procedure results in erroneously high serum prothrombin values, due apparently to the fact that thrombin is formed more rapidly from prothrombin in serum than it is from prothrombin in plasma. De Vries, Alexander and Goldstein (14) have suggested that the difference in prothrombin convertibility in plasma and serum, as observed in the one-stage method, is due to the elaboration in serum of an accelerator of prothrom-

bin conversion. This factor, as well as other differences in technique, may account for these apparently divergent results.

#### SUMMARY

The platelet requirements for clotting in dog and human blood have been compared. The rate of prothrombin utilization was used to indicate the clotting capacity of plasmas containing varied numbers of platelets. The results indicate that in both plasmas platelets are present in great excess over minimal requirements, and that below critical platelet levels, clotting is impaired. Dog plasma requires more platelets for a normal clotting rate than does human plasma. In terms of platelet count, the requirements of dog plasma are about 1.7 times greater than they are in human plasma; in terms of platelet volume, the requirements are about 3 times greater.

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# EVOLUTION OF A PROTHROMBIN CONVERSION ACCELERATOR IN STORED HUMAN PLASMA AND PROTHROBIN FRACTIONS<sup>1</sup>

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THE prothrombic activity of plasma, as measured by the one-stage method, decreases as plasma ages (1-3) because of deterioration of 'labile factor' necessary for the rapid conversion of prothrombin to thrombin by thromboplastin plus calcium. When, however, determinations are made by a modified procedure in which the test plasma is first diluted with *prothrombin-free fresh* plasma, the aged plasma shows *increased* prothrombic activity (3). Similar observations have been recorded by others (4-6).

From the data presented below it appears that the phenomenon is referable to the evolution during storage of a factor which can accelerate prothrombin conversion. This substance, similar to or identical with a prothrombin conversion accelerator which is elaborated during coagulation (7), can be separated from stored plasma by adsorbing it with BaSO<sub>4</sub> from which it can be eluted by sodium citrate.

## METHODS

Oxalated human plasma (1 volume of 0.1M sodium oxalate to 9 volumes of blood), prepared from blood centrifuged at 2000 rpm for 10 minutes, was stored at 3 to 5° C. At intervals prothrombic activity was determined both by the one-stage method of Quick (8), and by the modification of Rosenfield and Tuft (9) in which prothrombin-free plasma is used as diluent. The latter was prepared from oxalated plasma pooled from at least 5 normal subjects. In both procedures commercial thromboplastin (Difco) was used.

Fresh and stored plasma samples were also treated in the following manner: powdered BaSO<sub>4</sub> (C.P.) was added, the mixtures were shaken and kept at 37° C. for 15 minutes during which time they were frequently agitated, and then centrifuged at 3000 rpm for 30 minutes. The supernatant was separated; the BaSO<sub>4</sub> was washed twice with an equivalent volume of sodium acetate buffer (0.02M, pH 5.2) and eluted (once or twice) with sodium citrate solution (5% in physiological saline) which totaled in volume that of the original plasma.

The prothrombic activities of the supernatants and eluates were determined in the usual manner after diluting with fresh prothrombin-free normal plasma. The prothrombin conversion accelerator was measured by mixing these fractions with equal volumes of fresh whole plasma and computing the difference between the observed prothrombic activities of the mixtures and the sum of the activities of the components determined separately, employing the same dilution technique with prothrombin-free plasma.

## RESULTS

The data, recorded in figure 1, confirm the fact that as plasma ages its prothrombic activity, as measured on undiluted whole plasma, decreases progressively.

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However, when determinations are made using fresh prothrombin-free plasma as diluent, prothrombic activity increases during storage at refrigerator temperature until the aged plasma becomes approximately two or three times as active as the original unstored plasma. Thereafter, the activity slowly declines. The 'hyperreactivity' is not demonstrable in plasma which ages at room or body temperatures.

The interval of storage required for the development of increased prothrombic activity varies widely. In some instances the change becomes evident within 24 or 48 hours; in others, two weeks or more are necessary (table 1). When citrate (one part of 2.5 % sodium citrate solution to 9 parts of blood) is used instead of oxalate as anticoagulant the appearance of hyperreactivity is delayed. Also, as has been observed previously by others (10) and by us (3), the decrease in whole plasma prothrombic activity is retarded.

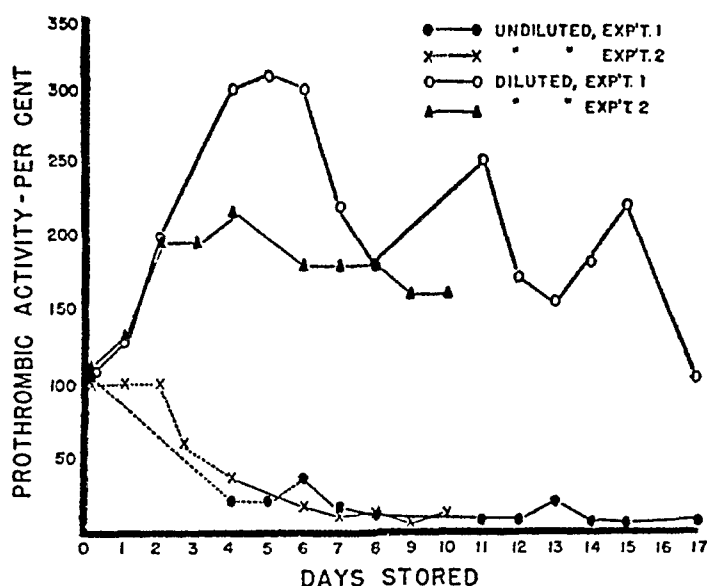


Fig. 1. PROTHROMBIC ACTIVITY OF OXALATED HUMAN PLASMA stored at 4°-5° C. Determinations made at intervals on the same plasma by the orthodox one-stage procedure on whole plasma (undiluted) and by the dilution technique (diluted) employing fresh BaSO<sub>4</sub> plasma as diluent.

Prothrombin can be adsorbed from plasma by BaSO<sub>4</sub> (9). We have found that it can be eluted from this adsorbing agent by sodium citrate. The 'hyperreactive' prothrombin of stored plasma behaves similarly. When plasma is adsorbed at intervals during storage with 25 mg. BaSO<sub>4</sub> per cc. and the adsorbate eluted with sodium citrate, the prothrombic activities of the eluates parallel those of the unadsorbed parent plasma (fig. 2). The supernatants from the BaSO<sub>4</sub> adsorption show little or no activity (fig. 2).

An eluate from the BaSO<sub>4</sub> adsorbate obtained from a 'hyperreactive' (166% of normal prothrombic activity) stored plasma exhibited only 10 per cent prothrombic activity and contained 31.1 micrograms of nitrogen derived from 1 cc. of plasma (table 2). However, when it was mixed with fresh plasma, the observed activity of the mixture was almost threefold the sum of the activities of the components. Separation thus of a plasma fraction, poor in prothrombin, yet capable of enhancing the prothrombic activity of fresh plasma to which it was added, indicates that the

'hyperreactivity' which develops in stored plasma is due to the evolution of a prothrombin conversion accelerator and not to deterioration of an inhibitor.

Prothrombin-rich fractions derived from fresh plasma by BaSO<sub>4</sub> adsorption and citrate elution also show increasing activity during storage (table 3). In contrast to some of the parent plasmas which were relatively slow in becoming hyperreactive the activity of the eluates increased within 24 hours (table 1). It should be noted that determinations on the eluates and the parent plasmas were made side by side, after both were adjusted to contain equal amounts of oxalate and citrate. The supernatants, very low in activity originally, remained unchanged. This indicated that the precursor of the accelerator which evolves during storage is adsorbed along with the prothrombin.

TABLE 1. PROTHROMBIC ACTIVITY OF PLASMA AND PLASMA FRACTIONS DURING STORAGE AT 3-5° C.

PLASMA FROM SUBJECT	TIME REQUIRED FOR HYPERREACTIVITY TO APPEAR, DAYS		PROTHROMBIC ACTIVITY <sup>1</sup>			
	Whole Plasma	Eluate from BaSO <sub>4</sub> Adsorption	Whole Plasma		Eluate from BaSO <sub>4</sub> Adsorption	
			<i>Initial</i>	<i>Maximal</i> <sup>2</sup>	<i>Initial</i>	<i>Maximal</i>
<i>A</i>	1		80	200(4)		
<i>B</i>	1		105	190(4)		
<i>C</i>	10		75	190(15)		
<i>D</i>	4		66	230(7)		
<i>ABCD</i> (pool)	1		78	230(7)		
<i>E</i>	15		59	140(15)		
<i>E</i> <sub>2</sub> <sup>3</sup>	>7	1	85		49	79
<i>F</i>	10		71	130(15)		
<i>F</i> <sub>2</sub> <sup>3</sup>	>8	1	85		68	108
<i>G</i>	4		63	170(7)		
<i>G</i> <sub>2</sub> <sup>3</sup>	5	1	75	140(6)	75	105

<sup>1</sup> Percentage of normal.

<sup>2</sup> Figures in parentheses denote the day of storage at which maximal activity was first observed.

<sup>3</sup> Plasma obtained from subjects *E*, *F* and *G* on other days.

It is possible that an accelerator might not be evident in the aged 'hypoprothrombinemic' plasma because its initial prothrombin content might be so small that an enhancement in activity might not be striking. Under such circumstances, addition of prothrombin in the form of fresh normal plasma or prothrombin-rich fractions should result in a greater prothrombic activity than would be expected on the basis of the added prothrombin alone. When prothrombin was thus added to the deprothrombinated stored plasma, no enhancement could be observed; the activity of these mixtures was equal to the sum of the activities of the components determined separately. When the prothrombin was only partially removed by adsorbing with smaller amounts of BaSO<sub>4</sub>, the 'hypoprothrombinemic' plasma (30% of normal) showed some rise in activity during storage (fig. 3) although less than the whole parent plasma.

Dicumarol-induced hypoprothrombinemia was also studied. Three patients, treated with this drug for impending or actual myocardial infarction, had between 5

and 12 per cent prothrombin. During storage the plasmas of two showed some increase in activity, the earliest change appearing after 14 days (table 4). However, when prothrombin was added in the manner described above, little, if any, further acceleration was demonstrable.

Thus, plasma rendered markedly hypoprothrombinemic either by  $\text{BaSO}_4$  adsorption or by dicumarol administration does not become strikingly 'hyperreactive' during storage or develop the ability to accelerate the conversion of added prothrombin. Similar results were obtained in a patient with hypoprothrombinemia due to severe hepatic cirrhosis (table 4). It is not clear whether this is referable to reduction of prothrombin per se or to simultaneous inadequacy of a non-prothrombin precursor of the accelerator. There is evidence that dicumarol lowers, besides prothrombin, a substance which affects prothrombin conversion (11, 12). Also, the patient with liver disease seemed to be deficient not only in prothrombin but also in

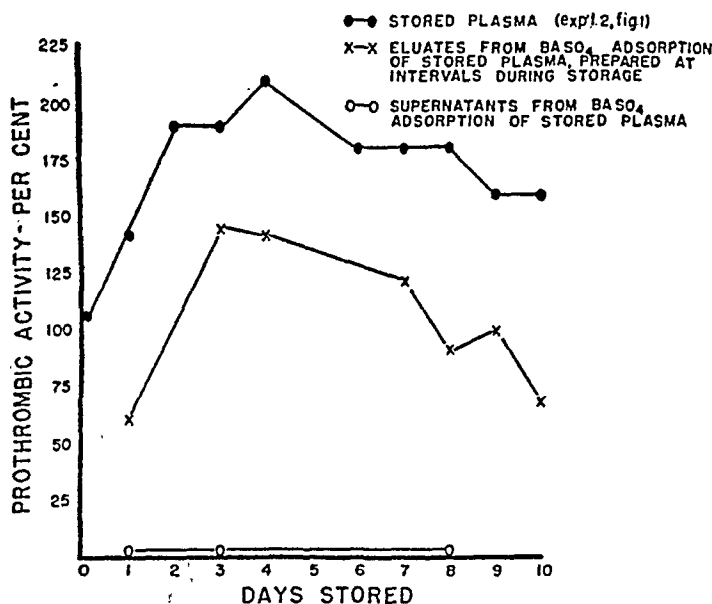


Fig. 2. FRACTIONATION OF NORMAL OXALATED PLASMA at intervals during storage; separation of 'hyperreactive' prothrombin by adsorption with  $\text{BaSO}_4$  and elution with sodium citrate solution.

plasma factors important in prothrombin conversion since the prothrombic activity of her whole plasma (14% of normal) was far less than that (33%) obtained with the dilution technique employing *prothrombin-free normal* plasma. Observations were also made on a 5-week-old patient with congenital hypoprothrombinemia<sup>2</sup> whose plasma prothrombic activity was 1 to 2 per cent of normal. During 6 days' storage it did not change appreciably nor did any accelerator evolve. Unfortunately, further observations could not be made since the patient died.

Platelets are not required for evolution of the accelerator. Normal oxalated plasma was rendered platelet-'free'<sup>3</sup> by centrifugation for 15 minutes at 15,000 rpm in a Type 2 international centrifuge with multi-speed attachment, kept in a constant

<sup>2</sup> We are grateful to Dr. Charles A. Janeway, Physician-in-Chief of the Children's Hospital, Boston, for his cooperation in making this subject available for study. Details will be reported in a separate communication.

<sup>3</sup> Counts made on the centrifuged plasma revealed 10,000 platelets or 'platelet bodies' per cu. mm. of plasma.

temperature room at 6°C. During storage the plasma developed increased prothrombic activity, parallel with the non-centrifuged plasma (fig. 4). The blood had been handled throughout in apparatus coated with silicone<sup>4</sup> until the plasmas were placed in ordinary glass for storage. Also, the accelerator could be separated from the aged platelet-free plasma by BaSO<sub>4</sub> adsorption and subsequent elution. Furthermore, plasma from a patient with thrombocytopenic purpura secondary to acute leukemia (platelet count = 30,000 per cu. mm. of blood) became hyperreactive during storage. That the platelets cannot, therefore, be implicated in evolution of the accelerator is in contrast to the interpretations of others (6, 14) who attributed the prothrombic hyperreactivity of stored plasma to the products of platelet lysis.

The question also arises whether exposure to a 'foreign' surface is required. Plasma derived from blood taken with siliconized apparatus and stored in siliconized

TABLE 2. DEMONSTRATION OF PROTHROMBIN CONVERSION ACCELERATOR IN A PLASMA FRACTION FROM "HYPERREACTIVE" STORED PLASMA

PROTHROMBIN MIXTURE CONTAINING:				PROTHROMBIN		
Stored Plasma (S.P.), Eluate (E) or Supernate (S)	Whole Fresh Plasma	Saline	Diluent: Proth.-Free Fresh Plasma	Time	Activity <sup>1</sup> Found	Activity Expected
<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>sec.</i>	<i>%</i>	<i>%</i>
S.P.—1	0	0	9	20.9	166	
E. <sup>2</sup> —3	0	0	7	69.0	10	
E. <sup>2</sup> —1	1	0	18	21.6	154	53
S.—1	0	0	4	33.8	34	
S.—1	1	0	18	30.3	76	65
0	1	1	18	41.8	96	

Pooled oxalated plasma stored at 4°–5°C. for 14 days, then adsorbed with 25 mg. BaSO<sub>4</sub>/cc. The BaSO<sub>4</sub> was then eluted with 5% sodium citrate in 0.9% saline solution; final volume was that of original plasma.

<sup>1</sup> Corrected for dilution of prothrombin mixture with prothrombin-free fresh plasma.

<sup>2</sup> Eluate contained 31.1 µg. N/cc.

vessels becomes equally hyperreactive at the same time as plasma from the same individual handled throughout in ordinary glass.

The prothrombin accelerator evolves also in stored hemophilic plasma. Of 5 hemophiliacs studied the plasmas from 2 showed increasing activity within 24 to 48 hours; in the others, a much longer time was required before any change was demonstrable. These phenomena could not be correlated with the clotting time of whole blood nor were they affected by accelerating coagulation with intravenous infusions of normal plasma (150–180 cc.) before obtaining the hemophilic plasma for storage.

On the theory that small amounts of thrombin, slowly evolved from prothrombin during storage, might be required for elaboration of the accelerator from an inert precursor (see later in discussion regarding conversion of plasma Ac-globulin to the serum type by thrombin) 0.1 and 0.2 units of thrombin (Parke, Davis topical thrombin) were added to 2.0 cc. of fresh chilled oxalated hemophilic plasma which alone showed delayed increase in prothrombic activity upon aging. The addition of throm-

<sup>4</sup> General Electric Dry Film #9987 used according to the technique of Jacques *et al.* (13).

bin did not accelerate the appearance of hyperreactivity. Small fibrin shreds and thin clots were observed in 24 hours, but the amount of fibrinogen thus removed had no demonstrable influence on the prothrombin time.

#### DISCUSSION

Changes in plasma prothrombic activity during storage have been studied by many workers. Non-uniformity of methods has led to divergent results and interpretations. Clearly the activity, as determined on whole plasma by the one-stage technique, declines after the first few days of storage, due to deterioration of a labile component which is present in fresh plasma (3).

When determinations are made on mixtures of the stored, with prothrombin-free fresh, plasma, the activity is found to increase progressively until, at its height, it may be two or three times the initial value. Enhanced reactivity of fibrinogen to

TABLE 3. PROTHROMBIC ACTIVITY OF PLASMA FRACTION DURING STORAGE AT 4-5°C.

DAYS STORED	PROTHROMBIC ACTIVITY—PER CENT <sup>1</sup>			
	15 mg. BaSO <sub>4</sub> Adsorption		25 mg. BaSO <sub>4</sub> Adsorption	
	<i>Eluate</i>	<i>Supernatant</i>	<i>Eluate</i>	<i>Supernatant</i>
0	70	10.0	120	0
1	140	10.5	140	0
2	115	9.0	155	0
4	165	9.0	200	0
7	128	11.4	170	0
8	126	10.8	190	0
9	170	8.0	180	0
10	158	—	158	—

Aliquots of oxalated plasma from one subject adsorbed with 15 mg. and 25 mg. BaSO<sub>4</sub> respectively. Prothrombic activities determined on supernatant plasmas, and on eluates obtained from the BaSO<sub>4</sub> by elution with 5% sodium citrate in saline.

<sup>1</sup> On basis of normal plasma containing 100% prothrombic activity

thrombin, one of the proposed explanations for this phenomenon (4), can be readily dismissed: the hyperreactivity of aged plasma is measured in a mixture in which 90 per cent or more of the clottable fibrinogen is provided by fresh prothrombin-free plasma. Furthermore, plasma fractions devoid of fibrinogen become hyperreactive during storage. Finally, we have found (15) that stored plasma becomes less clottable by standard solutions of thrombin.

Increasing prothrombic activity may reflect deterioration of an inhibitor such as an antiprothrombin, antithrombin or antithromboplastin. This explanation is also untenable since a prothrombin conversion accelerator can be separated from hyperreactive stored plasma.

Banfi *et al.* (6) attribute the hyperreactivity of stored plasma to a prothrombin 'sensitization,' whereby, under the influence of deteriorating platelets, prothrombin undergoes a molecular alteration yielding a product more rapidly convertible to thrombin by thromboplastin plus calcium. This interpretation, which resembles Bordet's (16) concept of proserozyme (less active prothrombin) being converted

during coagulation to serozyme (more active prothrombin), can be excluded also by the fraction obtained from stored plasma, which was itself very low in prothrombic activity but, nevertheless, could markedly accelerate thrombin evolution from added prothrombin.

The prothrombic 'hyperreactivity' of stored plasma is best explained by slow elaboration of a factor which activates, accelerates or otherwise acts as an ancillary agent in the evolution of thrombin in the presence of thromboplastin plus calcium. Formation of similar substances has also been observed during blood coagulation (17, 18). Ware *et al.* (17) have reported on *serum* Ac-globulin which arises, under the influence of small amounts of thrombin, from a relatively inert precursor, *plasma* Ac-globulin. During storage slow conversion of prothrombin to thrombin may occur despite the presence of anticoagulant, thus providing the conditions necessary for transforming plasma Ac-globulin into the serum type. It will be recalled, however,

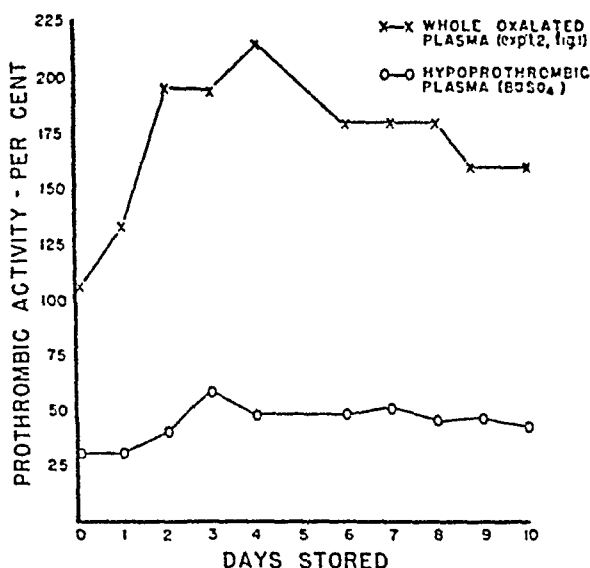


Fig. 3. PROTHROMBIC ACTIVITY, during storage at 4°-5° C., of BaSO<sub>4</sub> adsorption induced 'hypo-prothrombic' plasma and the parent plasma.

that the addition of small amounts of thrombin to hemophilic plasma failed to hasten the appearance of increased prothrombic activity.

Milstone (19) described a prothrombin convertor, thrombokinase, which evolves from an inert precursor, prothrombokinase, during the first stage of coagulation. Calcium is required for the transformation; whether it can proceed in oxalated plasma is unknown. It is unlikely that the accelerator which evolves in stored plasma is thrombokinase because prothrombokinase is not adsorbed by BaSO<sub>4</sub> (19) whereas the precursor of the accelerator which evolves in stored plasma is adsorbed.

A prothrombin conversion accelerator which arises during coagulation has also been demonstrated in human serum by de Vries *et al.* (7). It is relatively stable, in contrast to the extreme lability of Ac-globulin in human serum (10), can be adsorbed quantitatively by BaSO<sub>4</sub> or BaCO<sub>3</sub> and eluted by citrate solutions (20), and it is greatly reduced in serum from hypoprothrombinemic blood.<sup>5</sup> Its remarkable re-

<sup>5</sup> In congenital hypoprothrombinemia, as well as that induced by dicumarol (21).

semblance in these respects to the accelerator which evolves in stored plasma suggests that as plasma ages very slow 'coagulation' occurs during which the 'serum' prothrombin conversion accelerator evolves. This would explain the deposition of fibrin clots during storage despite the presence of anticoagulant.

That the accelerator cannot be detected by determinations on *whole* stored plasma<sup>6</sup> suggests that labile factor or some other plasma component similarly labile is necessary for its activity. During the first several weeks of storage, prothrombic activity, as measured by the orthodox one-stage technique, is the resultant of prothrombin concentration, unaltered labile factor and evolved prothrombin conversion accelerator. This must be considered in assays of labile factor based upon restoring prothrombic activity to stored plasma by adding fresh plasma or plasma fractions (5).

Progressive increase in the activity of aging prothrombin-rich plasma derivatives brings to mind the report of Ware and Seegers (22) on 'regeneration' of purified bovine prothrombin in the presence of thrombin. The question arises whether their observations are referable to evolution of a factor which favorably affects the yield as well as the velocity of thrombin formation.

TABLE 4. PROTHROMBIC ACTIVITY OF STORED HYPOPROTHROMBINEMIC OXALATED PLASMA

<i>Days Stored</i>			PROTHROMBIC ACTIVITY—PER CENT						
	0	1	3	7	14	17	21	24	
SUBJECT	<i>Dicumarolized subjects</i>								
<i>Mrs. D.</i>	9	5	6	12	22	18			
<i>Mrs. B.</i>	12	16	9	27	20	22			
<i>Mr. S.</i>	12	15	7	12	13	14			
	<i>Subject with cirrhosis of liver</i>								
<i>Mrs. H.</i>	33		35	36	28	44	43	35	

Changes in activity of prothrombin-rich fractions deserve careful consideration in the purification of prothrombin when guided by the one-stage technique. Also, prothrombin assays by the two-stage procedure must be viewed with caution until one can be sure that an accelerator evolving in a prothrombin fraction does not increase the yield of thrombin. In this connection there should be mentioned the recent observations of Lewis and Ferguson (23) that no limit is reached in the amount of thrombin obtained from a given quantity of purified prothrombin to which increasing amounts of Ac-globulin are added.

Little can be said regarding the precursor of the accelerator. Platelets can be excluded by the experiments on thrombocytopenic plasma and on normal plasma stored in siliconized vessels. Our findings on plasma rendered prothrombin-deficient by dicumarol or by adsorption with BaSO<sub>4</sub> suggest that the plasma prothrombin concentration must be at least 30 per cent of normal before substantial amounts of the accelerator can evolve. However, this is probably not the sole requisite, as indicated by observations on the cirrhotic patient whose plasma, although it contained 30 to 40 per cent prothrombin, failed to become hyperreactive. This may have been related

<sup>6</sup> Schilling *et al.* (13) report 'hyperreactivity' of stored citrated plasma within the first 5 days of storage. The increases in activity, 10-12 per cent, is only suggestive in view of limitations in the method they employed.

to the inadequacy of non-prothrombin factors important in the evolution of thrombin from prothrombin,<sup>7</sup> which was observed in this subject.

Thus, two important changes in plasma clotting components have been demonstrated during storage: elaboration of an accelerator which favorably affects the velocity of prothrombin conversion, and deterioration of labile factor, which adversely influences this reaction. The question arises whether these phenomena are related. At first glance it appears that they are not since prothrombic hyperreactivity may appear in some instances before any deterioration of labile factor is detectable from determinations of the prothrombin time on whole plasma. However, as has already been mentioned the prothrombin time of plasma, at any point during storage, reflects the concentration of prothrombin, labile factor and the prothrombin conversion accelerator which evolves. Therefore, normal prothrombin time on the second, third or fourth day of storage does not necessarily exclude some deterioration of labile factor. Some loss in this component may have occurred, yet because of

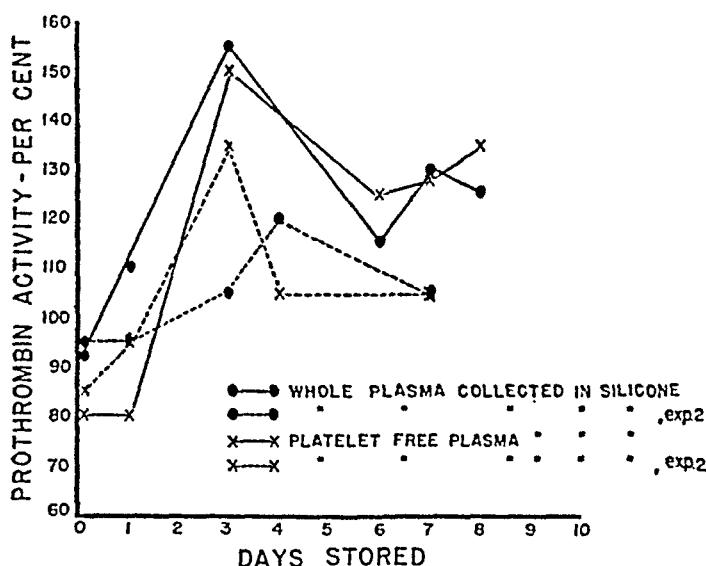


Fig. 4. PROTHROMBIN ACTIVITY, during storage at 4°-5° C., of platelet-rich and platelet-poor (centrifuged) oxalated human plasma.

elaboration of some accelerator the prothrombin time need not be altered. Accordingly, deterioration of labile factor and evolution of the accelerator during storage may be interrelated.

It is also noteworthy that prothrombin hyperreactivity consequent to aging cannot be demonstrated unless a component found in *fresh* plasma is simultaneously provided. This suggests that labile factor, or some other plasma substance similarly labile, is essential for the activity of the accelerator in speeding thrombin evolution. The same is true of the prothrombin conversion accelerator found in serum (7).

That the accelerator appears earlier in a prothrombin rich fraction than in the parent plasma suggests that its evolution may be retarded by the presence of one or more of the plasma proteins, or that the manipulation of fractionation may, somehow, render the precursor more susceptible to transformation.

<sup>7</sup> Insufficiency of plasma Ac-globulin has been induced in dogs by injuring the liver with chloroform, resulting in an elevated prothrombin time (24).

Why hyperreactivity does not occur in plasma stored at room or body temperature in contrast to refrigerator temperature is obscure.

#### CONCLUSIONS

The prothrombic activity (one-stage) of normal plasma or prothrombin-rich fractions obtained by adsorption with  $\text{BaSO}_4$  and elution with sodium citrate increases during storage at refrigerator temperature. This does not occur at room or body temperature. In some plasmas prothrombic hyperreactivity appears within 24 or 48 hours; in others it develops after two or more weeks of storage. In the latter instances, prothrombin-rich fractions become hyperreactive much earlier than the parent plasmas.

The phenomenon is attributable to evolution of an agent which accelerates the conversion of prothrombin to thrombin by thromboplastin plus calcium. The agent as well as its precursor(s) can, like prothrombin and the prothrombin conversion accelerator of serum, be adsorbed by  $\text{BaSO}_4$  and eluted with sodium citrate. It arises in hemophilic plasma, thrombocytopenic plasma, and in normal plasma handled entirely in siliconized apparatus. The accelerator cannot be demonstrated in the absence of a labile factor present in fresh plasma.

Changes in stored dicumarolized plasma were not striking. Also, the accelerator did not evolve in plasma deprived of prothrombin by adsorption with  $\text{BaSO}_4$ , or in hypoprothrombinemic plasma from a patient with severe hepatic cirrhosis. Its possible relationship to serum Ac-globulin, or the prothrombin conversion accelerator of serum, is discussed.

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# THE IN VITRO RELEASE OF HISTAMINE FROM THE BLOOD CELLS OF SENSITIZED RABBITS: RELATIONSHIP TO BLOOD COAGULATION MECHANISMS<sup>1</sup>

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HISTAMINE is released from the blood cells into the plasma when antigen is added *in vitro* to whole blood from the sensitized rabbit. This was first demonstrated by Katz (1) and has been considered by him and other workers (2) to be an *in vitro* anaphylactic type of reaction. It is well known also that histamine is released from the blood cells when rabbit blood is allowed to coagulate. In a study of the mechanism of the *in vitro* anaphylactic reaction, the possibility of participation by different components of the blood coagulation system must therefore be considered. In this paper are reported the results of our studies on the relationship between the anaphylactic histamine release mechanism and certain components of the blood coagulation system. Our evidence indicates that prothrombin, thrombin, thromboplastin and Ac-globulin are not involved in the anaphylactic histamine release. On the other hand, the mechanism of this reaction has some properties in common with the blood coagulation mechanism.

## EXPERIMENTAL

Rabbits, 2 to 4 kg. in weight, were sensitized by the intramuscular injection of 0.2 ml. of antigen emulsion into each of 10 different sites on the same day. The antigen emulsion was of the Freund type (3) with constituents as follows: 7 mg. of dry heat-killed saprophytic acid-fast bacterial cells, 4 ml. heavy mineral oil, 2 ml. aquaphor, and 8 ml. of fresh egg white. In 10 to 14 days after the injection of antigen, the rabbits were exsanguinated through the carotid artery by means of a coated cannula, under local anesthesia. For each 50 ml. of blood collected 0.1 ml. of heparin solution (1000 units/ml.) was added. All glassware in which whole blood was handled was coated with either General Electric Dri-film 9987 or Dow-Corning Pan Glaze. The latter film is very durable and the glassware can be used repeatedly without being recoated.

Crystalline trypsin and crystalline soy trypsin inhibitor were kindly supplied by Dr. M. Kunitz. For some experiments the trypsin inhibitor preparations were made in our laboratory according to the procedure of Kunitz (4). The sodium salt of heparin, 100 units per milligram, kindly supplied by Dr. Ira B. Cushing of these laboratories, was used in all experiments. A 1 per cent solution of heparin was made up in saline *without a preservative*. The chloride salts of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  were used as the sources of these ions.

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All *in vitro* histamine release reactions were carried out as follows. Constituents of the reaction in isotonic solution were pipetted into a heavy duty 12 ml. conical centrifuge tube and the volume was diluted to 2.6 ml. with saline. To this solution 2 ml. of whole blood was added, followed by thorough mixing, and then 1 ml. of a 1:100 dilution of whole egg white in saline was added. The contents of each tube were mixed well; the tubes were placed in a 37°C. water bath for 20 minutes and then in an ice bath. Blood cells were removed by centrifugation for 15 minutes in a refrigerated centrifuge. A 4.5 ml. aliquot of plasma was carried through the histamine purification procedure of McIntire, Roth and Shaw (5). At the end of the histamine purification, the cotton succinate eluates were not neutralized; the acid eluates were evaporated to dryness at reduced pressure and the histamine was determined by the chemical method of McIntire *et al.* (6). In our data the concentration of heparin,

TABLE 1. IN VITRO HISTAMINE RELEASE FROM BLOOD CELLS OF DICUMAROL-TREATED SENSITIZED RABBITS

RABBIT NO.	CLOTTING TIME	HISTAMINE RELEASED	
		$\mu\text{g/ml. of blood}$	% of total
	<i>minutes</i>		
920	10	1.79	72
921	>25	1.75	54
922	20	1.44	41
923	12.5	1.4	29
924	24	1.2	42
926	18.5	3.21	68
Normals untreated	2 to 4	0.5 to 4.3	27 to 79

phenol, oxalate, citrate,  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  and trypsin inhibitor are given in terms of the final dilution in the *in vitro* reaction. The concentration per ml. of blood would be 2.5 times the concentration indicated.

#### RESULTS AND DISCUSSION

The *in vitro* histamine release by antigen can be carried out without any noticeable blood coagulation if silicone-coated glassware and a very small amount of heparin are used. However, this fact does not exclude the possibility that some of the enzymatic components of the coagulation mechanism might participate in the histamine release reaction. One of our first approaches to this question was the treatment of sensitized rabbits with high doses of dicumarol to decrease the prothrombin content of the blood as much as possible just short of fatal treatment.

The animals were given 10 mg. of dicumarol per kilo intravenously each day (usually for 5-6 days) until the blood-clotting time was greatly prolonged. They were then exsanguinated and the degree of anaphylactic *in vitro* histamine release was determined. The data of table 1 indicate that the heavy dicumarol treatment did not affect the anaphylactic histamine release mechanism. There is no relationship between the clotting time of the blood and the percentage of the total histamine released by antigen. The percentage of total histamine released from blood of both dicumarol-treated rabbits and untreated rabbits is essentially the same. Since it is well known that dicumarol treatment sufficient to prolong markedly the blood-clotting time

greatly decreases the prothrombin content of the blood, we may conclude that a great decrease in the prothrombin content does not impair the anaphylactic histamine release mechanism.

The inhibition of the *in vitro* anaphylactic reaction by heparin was first reported by Dragstedt *et al.* (2). The amount of heparin they used to inhibit the reaction (0.12%) was much higher than the amount required to prevent coagulation. We have determined the inhibition of histamine release by various levels of heparin and the data are shown in figure 1. The heparin concentrations indicated represent the concentration over and above the 0.008 mg/ml. which was used in all experiments

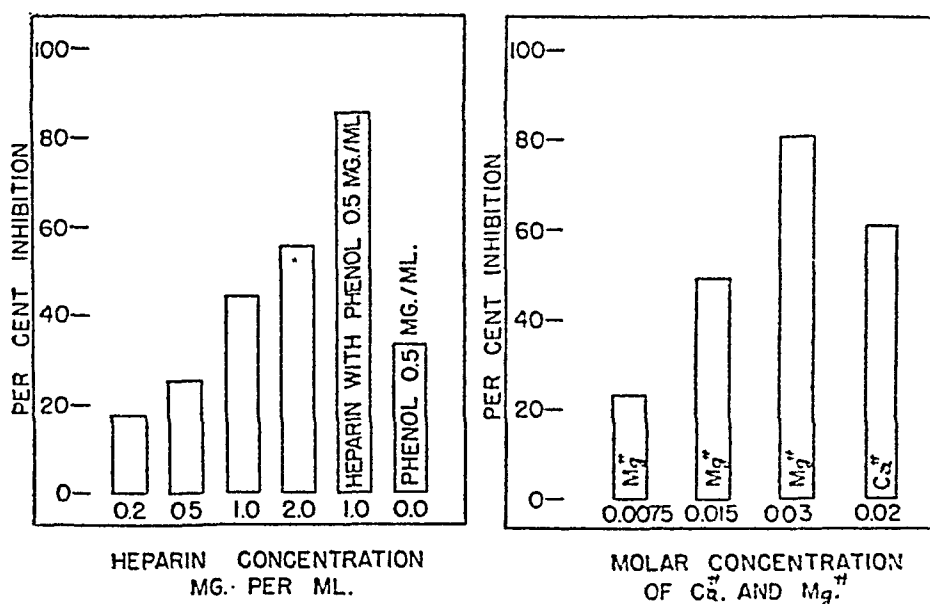


Fig. 1 (left). HEPARIN inhibition of the *in vitro* 'anaphylactic' histamine release.

Fig. 2 (right). INHIBITORY effect of  $Mg^{++}$  and  $Ca^{++}$  on the *in vitro* release of histamine by antigen.

to prevent blood clotting. These data are an average of several experiments. The deviation from the average values is not great when the histamine release in the controls is average or above. In experiments with very poor histamine release the inhibition by the lower levels of heparin is greater percentage-wise. These data indicate that the amount of heparin necessary to give a 55 per cent inhibition of the anaphylactic histamine release is at least 250 times the amount required to prevent blood clotting for several hours under these conditions, and approximately 1000 times the amount of heparin required to inhibit markedly the action of thrombin and thromboplastin in more purified systems (7). The weak inhibition of the histamine release reaction by heparin very strongly suggests that neither thrombin nor thromboplastin is important in the *in vitro* anaphylactic reaction. The data on the inhibition by *phenol* and *heparin + phenol* are included to point out the potential error had we used a commercial heparin containing phenol as a preservative. One mg/ml. of heparin with the usual amount of phenol used as a preservative gives a much greater inhibition than 2 mg/ml. of heparin without phenol. In fact, milligram for milligram the phenol is a more potent inhibitor than is heparin.

Further evidence against the participation of thromboplastin and Ac-globulin

in the histamine release reaction is the fact that the soy bean trypsin inhibitor fails to inhibit this reaction (table 2). MacFarlane (7) found that a concentration of 0.01 mg/ml. of soy trypsin inhibitor markedly inhibited the conversion of prothrombin to thrombin. Our data show that even 1.6 mg/ml. of the trypsin inhibitor does not significantly inhibit histamine release by antigen, while only 0.4 mg. of inhibitor per ml. almost completely inhibits histamine release by an optimal concentration of trypsin. Since the soybean trypsin inhibitor is a potent inhibitor for both Ac-globulin and thromboplastin (8) these components of the blood coagulation system very likely do not participate in the *in vitro* anaphylactic histamine release.

The failure of soy trypsin inhibitor to inhibit the histamine release by antigen is also concrete evidence against the popular theory that the histamine release depends

TABLE 2. EFFECT OF SOYBEAN TRYPSIN INHIBITOR ON HISTAMINE RELEASE BY ANTIGEN AND BY TRYPSIN

HISTAMINE RELEASE AGENT	SOY TRYPSIN INHIBITOR mg/ml.	% INHIBITION
Antigen	0.8	7
	1.6	7
Trypsin		
0.08 mg/ml.	0.4	95
0.15 mg/ml.	0.4	74

TABLE 3. OXALATE AND CITRATE INHIBITION OF HISTAMINE RELEASE BY ANTIGEN

INHIBITOR	MOLAR CON- CENTRATION	% INHIBITION	INHIBITOR	MOLAR CON- CENTRATION	% INHIBITION
Oxalate	0.002	22	Citrate	0.0017	42
	0.006	100		0.0051	96
	0.01	100		0.0068	100
				0.0085	100

upon the activation of the plasma protease, fibrinolysin. This point will be considered more completely in a later publication.

The histamine release mechanism has some properties in common with the blood coagulation system in being inhibited by oxalate, citrate,  $Mg^{++}$  and  $Ca^{++}$  as shown in figure 2 and table 3. The concentration of  $Mg^{++}$  which gives an 80 per cent inhibition of histamine release will infinitely prolong the clotting time of rabbit plasma (9), and the amount of  $Ca^{++}$  which will prolong the clotting time of rabbit blood to more than 60 minutes (9) gives a 60% inhibition of the histamine release. The concentrations of oxalate and citrate which are required for a 100 per cent inhibition of the histamine release reaction are of the same order as are required for effective anti-coagulant action.

There is, however, one important difference in the effect of citrate on the two mechanisms under consideration. While there is a slow inactivation of some component of the blood coagulation system under the influence of 0.02M citrate (10), there is a very rapid inactivation of the histamine release mechanism in a much lower

concentration of citrate (0.0068M). The data of table 4 show that when calcium chloride is added to citrated blood in only one minute after the citrate has been added, the inhibition by citrate is nearly 60 per cent irreversible. If the citrate is allowed to react with the blood for 20 minutes before the addition of  $\text{Ca}^{++}$ , the inhibition is 75 per cent irreversible. Attempts to reverse the oxalate inhibition resulted in coagulation, hemolysis and the release of histamine before antigen was added.

The fact that oxalate, citrate,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  inhibit the anaphylactic release of histamine does not necessarily mean that this reaction involves any part of the blood coagulation system. The rapid irreversible citrate inhibition of the anaphylactic

TABLE 4. IRREVERSIBILITY OF CITRATE INHIBITION OF HISTAMINE RELEASE BY ANTIGEN

MOLAR CONCENTRATION	PERCENTAGE INHIBITION		MOLAR CONCENTRATION	PERCENTAGE INHIBITION	
	1 min. <sup>1</sup>	20 min. <sup>1</sup>		1 min. <sup>1</sup>	20 min. <sup>1</sup>
Citrate 0.0068	97	100	Citrate 0.0068 CaCl <sub>2</sub> 0.006	57	69
Citrate 0.0068 CaCl <sub>2</sub> 0.004	56	79	Citrate 0.0068 CaCl <sub>2</sub> 0.008	74	76

<sup>1</sup>This indicates the length of time citrate was allowed to react with the blood before  $\text{Ca}^{++}$  was added.

histamine release indicates that the histamine release mechanism differs from the blood coagulation system with respect to the components affected by citrate.

#### SUMMARY

Thrombin, prothrombin, thromboplastin and Ac-globulin probably are not involved in the *in vitro* release of histamine by antigen from the blood cells of sensitized rabbits because: a) severe dicumarol treatment does not affect the histamine release, b) extremely large amounts of heparin only partially inhibit the histamine release, and c) soy bean trypsin inhibitor fails to inhibit the histamine release.

Oxalate, citrate,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  inhibit the histamine release reaction at approximately the same concentrations as are required for effective anticoagulant action. The histamine release mechanism is much more rapidly inactivated by citrate than is any part of the blood coagulation system.

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## COLORIMETRIC DETERMINATION OF LIPASE AND ESTERASE IN DOG'S SERUM<sup>1</sup>

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THE evidence has become increasingly convincing that there are two distinct esterolytic enzymes capable of splitting a wide variety of carboxylic acid esters (1-4). One enzyme, esterase, is abundant in liver, kidney, blood serum and pancreas, hydrolyzes esters of short chain fatty acids with greater facility than esters of long chain fatty acids and is inhibited by fluoride (5), atoxyl (6, 7), and taurocholate (8, 9). The other enzyme, lipase, is abundant almost exclusively in pancreas, hydrolyzes esters of long chain fatty acids ( $C_8$ - $C_{18}$ ), (2, 4) is inhibited by quinine (10) and specifically accelerated by taurocholate (4, 8). Fatty substances such as tributyrin are hydrolyzed by both enzymes (11). Even olive oil, which is considered to be an ideal substrate for lipase, is hydrolyzed to a slight extent by esterase.

These facts account for the lack of specificity possessed by current methods for measuring lipase, which utilize tributyrin (12), 'Tween' (13) or more reliably, olive oil, as substrates. The fatty acid produced by enzymatic hydrolysis is determined by titration with  $N/20$  sodium hydroxide (14). This is not an easy or convenient procedure to perform accurately, especially in the presence of serum protein and a heavy emulsion. A reliable method for measuring serum lipase would provide a valuable tool for the study of pancreatic disease.

A recent study of esterase and lipase activity of the tissues of several species by the use of three chromogenic substrates suggested the possibility of developing a convenient, sensitive and specific method for measuring serum lipase and esterase (4). It has been shown that two injections of mecholyl and eserine at 15-minute intervals produced a regular increase in the hydrolytic activity of dog serum upon olive oil (15). This technique has been modified by the use of acetic ( $C_2$ ), lauric ( $C_{12}$ ), and palmitic-stearic ( $C_{16}$ - $C_{18}$ ) acid esters of beta naphthol, instead of olive oil, as substrates (4). Following enzymatic hydrolysis, beta naphthol is coupled with tetrazotized diorthoanisidine to form a purple azo dye, which is extracted from the aqueous medium with ethyl acetate and measured in a photoelectric colorimeter (4). This method for serum lipase and esterase has been studied in dog's serum and forms the basis of this report.

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<sup>2</sup> Research fellow of the National Cancer Institute.

## METHOD

Venous blood (10 cc.) was taken from 11 mongrel dogs. Immediately following this, two subcutaneous injections at 15-minute intervals were made of acetyl beta methylcholine hydrochloride, and of eserine sulfate (0.05 mg/kg. body weight each). Blood samples were taken after 1 hour and again after 2 hours. In two experiments specimens were also taken 7 and 24 hours later. The clotted blood was centrifuged at 2000 rpm for 15 minutes, and the serum was diluted with water for the determination of esterase and lipase (table 1).

The synthesis of the substrates, beta naphthyl acetate, beta naphthyl laurate, and beta naphthyl palmitate-stearate, is given elsewhere (4, 16). Each substrate (10 mg.) was dissolved separately in acetone (2 cc. for the acetate or 10 cc. for the laurate or palmitate-stearate) and introduced through a submerged pipette into a swirling mixture of 20 cc. veronal buffer<sup>3</sup>, pH 7.4 and water (final volume 100 cc.). The final concentration of substrate was 0.1 mg/cc. To 5 cc. of each of these solutions a quantity of diluted serum (see table 1) was added. Incubation was then conducted for the time and at the temperature given in table 1. Different concentra-

TABLE 1

SUBSTRATE	SERUM DILUTION FACTOR	PERIOD OF INCUBATION	TEMPERATURE OF INCUBATION
		hr.	°C.
Beta naphthyl acetate.....	40	$\frac{1}{2}$	23-27
Beta naphthyl laurate.....	20	2	37.5
Beta naphthyl palmitate-stearate.....	10	24	37.5

tions and conditions for each substrate were required because of the widely differing rates of enzymatic hydrolysis of these esters. At the end of the period of incubation, 1 cc. (4 mg.) of a freshly prepared, cool solution of tetrazotized diorthoanisidine<sup>4</sup> was added and shaken into each tube. A purple azo dye formed immediately. Forty per cent trichloroacetic acid (1 cc.) was then added to break the protein-azo dye complex, and the pigment was extracted by shaking with 10 cc. ethyl acetate. The tubes were centrifuged for 15 minutes at 1500 rpm and 5 cc. of the clear, purplish-red organic layer was transferred to a colorimeter tube with a pipette, and measured with a photoelectric colorimeter (Klett) through a 540 m $\mu$  filter. From a calibration curve of pure beta naphthol, which is linear between 0.005 to 0.01 mg., color density was converted to milligrams and micromoles of naphthol.

In experiments in which inhibitors of the reaction or activators were studied, the diluted serum was incubated with 1 cc. of the appropriate agent for 30 minutes at room temperature before the substrate solution was added. These agents were

<sup>3</sup> Prepared (17) by mixing 66.5 cc. of a solution containing 10.3 gm. of sodium diethyl barbiturate in 500 cc. of distilled water with 33.5 cc. of an 0.1 M hydrochloric acid solution.

<sup>4</sup> Available commercially in powder form, containing 20% tetrazotized diorthoanisidine, 5% zinc chloride and 20% aluminum sulfate, under the trade name, Dupont Naphthanil Diazo Blue B. Provided through the courtesy of Dr. E. R. Laughlin, Dupont de Nemours and Co., Boston, Mass.

stored in the refrigerator in the following concentrations: sodium fluoride, 30 mg/cc.; sodium taurocholate, 2.2. mg/cc.; and quinine sulfate, 40 mg/cc.

## RESULTS

The sera of 11 dogs showed little change in ability to hydrolyze beta naphthyl acetate after injection of mecholyl and eserine. However, with naphthyl laurate and naphthyl palmitate-stearate, the sera of 7 dogs showed an appreciable increase in esterolytic activity after mecholyl and eserine, 2 showed a slight increase in enzymatic activity and 2 were unaffected. The data in 4 experiments which showed a significant elevation in enzymatic activity are given in table 2. These increases were more striking when the sera were exposed to sodium taurocholate prior to

TABLE 2. ESTEROLYTIC ACTIVITY OF DOG SERUM BEFORE AND AFTER INJECTION OF MECHOLYL AND ESERINE

SUBSTRATE	EXPER. NO.	MICROMOLES OF NAPHTHOL LIBERATED PER CC. OF SERUM/HOUR		
		Before Injection	1 Hour After Injection	2 Hours After Injection
Beta naphthyl acetate.....	3	39	41	41
	4	24	27	31
	9	75	92	92
	10	55	58	66
Beta naphthyl laurate.....	3	0.43	0.63	0.49
	4	0.28	0.42	0.42
	9	0.76	2.7	2.4
	10	0.49	0.77	0.63
Beta naphthyl palmitate-stearate.....	3	0.03	0.04	0.03
	4	0.00	0.01	0.01
	9	0.06	0.22	0.14
	10	0.03	0.10	0.05

incubation with the substrates (table 3). Under these conditions, the sera of 9 of 11 dogs showed marked increases in esterolytic power for all 3 esters after mecholyl and eserine injection (fig. 1). The rises in esterolytic power caused by sodium taurocholate were 12 to 228 per cent (acetate), 33 to 620 per cent (laurate), and 42 to 900 per cent (palmitate-stearate).

In 2 dogs followed for 24 hours after mecholyl and eserine injection (fig. 1), a slow fall in esterase and lipase activity was noted between 2 and 6 hours. Normal levels were reached within 24 hours.

Further evidence for the specificity of action of the enzyme which appeared in increased amount in the serum after mecholyl and eserine was provided by experi-

<sup>1</sup> Liver and pancreas were homogenized and diluted with water so that each cc. contained the following wet weight (1) of liver: 0.25-0.5 mg. for acetate substrate, 1.0 mg. for laurate, and 5.0 mg. for palmitate-stearate; and (2) of pancreas: 0.1-0.2 mg. for acetate and laurate, and 0.1 mg. for the palmitate-stearate.

ments with taurocholate, fluoride and quinine. Homogenates<sup>5</sup> of liver and pancreas were prepared and tested in exactly the same way as serum (table 4).

The esterolytic action of liver was inhibited by sodium taurocholate, whereas that of pancreas and serum (S<sub>1</sub>) was accelerated. Serum after injection of mecholyl

TABLE 3. ESTEROLYTIC ACTIVITY OF DOG SERUM ACTIVATED BY SODIUM TAUROCHOLATE BEFORE AND AFTER INJECTION OF MECHOLYL AND ESERINE

SUBSTRATE	EXPER. NO.	MICROMOLES OF NAPHTHOL LIBERATED PER CC. OF SERUM/HOUR		
		Before Injection	1 Hour After Injection	2 Hours After Injection
Beta naphthyl acetate.....	3	55	72	80
	4	30	85	98
	9	73	117	117
	10	68	117	117
Beta naphthyl laurate.....	3	3.5	6.6	8.5
	4	1.2	9.0	9.8
	9	7.6	9.7	9.8
	10	3.5	9.7	9.8
Beta naphthyl palmitate-stearate.....	3	0.08	0.27	0.35
	4	0.04	0.38	0.41
	9	0.15	0.41	0.41
	10	0.04	0.41	0.41

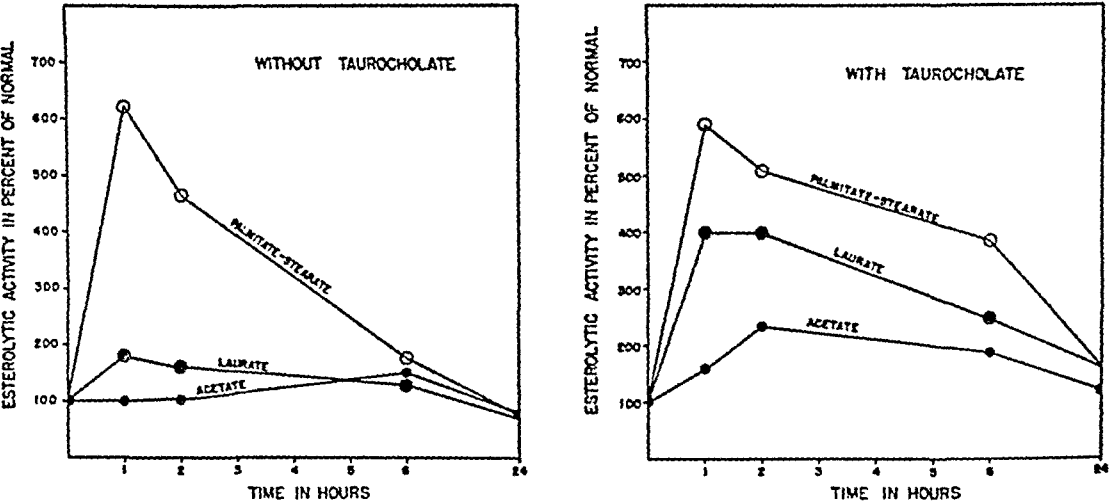


Fig. 1. THE FIRST GRAPH shows the esterolytic activity of dog serum on 3 substrates after the injection of mecholyl and eserine. The data is plotted in percentage of the activity of the pre-injection level and shows that the enzyme which appears in the serum hydrolyzes preferentially the higher fatty acid esters. The second graph shows the stimulating action of taurocholate on the hydrolytic activity of the enzyme (lipase) which appears in the serum after injection of mecholyl and eserine. The peaks of the rises were actually higher than shown (beyond range of the colorimeter). The greatest effect of taurocholate is shown with beta naphthyl laurate.

and eserine (S<sub>2</sub>) was accelerated even more, suggesting that the increased hydrolytic activity of serum was due to an enzyme (lipase) found in pancreas and not in liver.

Sodium fluoride inhibited markedly the action of the tissues and serum on the 3 substrates. However, when both taurocholate and fluoride were added, although inhibition of hydrolysis was still observed in liver and serum ( $S_1$ ), accentuation was observed with pancreas on all 3 substrates, and with serum ( $S_2$ ) on the laurate and palmitate-stearate esters. Thus the activating effect of taurocholate on lipase was apparent even in the presence of fluoride.

Quinine inhibited esterolysis, except for two instances of acceleration; i.e. pancreas on the acetate substrate, and liver on the palmitate-stearate substrate. With both taurocholate and quinine, the quinine effect was the same but more pronounced except for one instance of reversal; i.e. increased hydrolysis of naphthyl laurate by pancreas.

TABLE 4. EFFECTS OF CERTAIN AGENTS UPON ESTEROLYTIC ACTIVITY OF LIVER, PANCREAS, AND SERUM IN THE DOG<sup>1</sup>

REAGENTS	BETA NAPHTHYL ACETATE				BETA NAPHTHYL LAURATE				BETA NAPHTHYL PALMITATE-STEARATE			
	Liver	Pan-creas	Serum 1 <sup>2</sup>	Serum 2 <sup>2</sup>	Liver	Pan-creas	Serum 1 <sup>2</sup>	Serum 2 <sup>2</sup>	Liver	Pan-creas	Serum 1 <sup>2</sup>	Serum 2 <sup>2</sup>
Sodium taurocholate	oo	++	+	++	o	++	++	+++	o	++	+	++++
Sodium fluoride	ooo	ooo	ooo	ooo	ooo	ooo	ooo	ooo	oo	oo	ooo	ooo
Sodium fluoride and sodium taurocholate	oo	+	oo	oo	oooo	++	o	++	o	++	oo	++
Quinine	o	+	ooo	ooo	o	oooo	ooo	ooo	+++	oooo	ooo	ooo
Quinine and sodium taurocholate	ooo	++	oooo	ooo	oo	+	ooo	ooo	+++	oooo	oooo	oooo

<sup>1</sup> Symbols have the following meaning:

Inhibition Acceleration

o            + slight  
 oo          ++ strong  
 ooo        +++ marked  
 oooo      ++++ maximum

<sup>2</sup> Serum 1 refers to serum taken before injection of eserine and mecholyl, while serum 2 refers to serum taken 1 hour following injection.

#### DISCUSSION

The work of several investigators (1-4) indicated that the esterolytic activity of liver and serum differed from that of pancreas according to the type of substrate which was readily hydrolyzed by each. Other evidence for the specificity of pancreatic lipase was afforded by the experiments of Cherry and Crandall (14), and later by Nothman, Pratt and Benotti (18), in which an increase in the amount of enzyme able to split olive oil was demonstrated in the serum of the dog after pancreatic injury, whereas no increase in ethyl butyrase activity was demonstrated under these conditions. Similar changes were reported by Popper and Necheles (15) after injection of mecholyl and eserine. The experiments with the chromogenic substrates reported here confirm these observations.

Since esterase may hydrolyze olive oil to a slight extent and lipase may hydrolyze esters of short chain fatty acids, more conclusive proof of the specificity of the lipase activity in serum was needed. This was provided by the experiments with taurocholate and to a lesser degree with the other accelerators or inhibitors. Since taurocholate inhibits the esterolytic action of liver (esterase) and accelerates the activity of pancreas (lipase), acceleration of the esterolytic activity of serum, particularly after mecholyl and eserine injection, indicates that the enzyme appearing in the blood is pancreatic in origin (lipase). The taurocholate effect not only adds specificity to the serum lipase determination but increased sensitivity as well.

The strong hydrolytic action of serum on naphthyl acetate demonstrates esterase; the smaller hydrolytic action of serum on naphthyl laurate and palmitate-stearate demonstrates both esterase and lipase. Liver acts similarly. However, the constant accelerating effect of taurocholate upon serum and its inhibitory effect on liver indicates that lipase is present in normal serum but not in liver, within the limits of this method.

While human liver and pancreas act the same as dog liver and pancreas, human serum differs from dog serum in that lipase is not demonstrable by this technique in normal serum. The determination of serum lipase and esterase in man will form the subject of another communication (19).

#### SUMMARY

Methods for the colorimetric determination of esterase and lipase in the serum of dogs are given. Increase in lipase content of serum was demonstrated in dogs after injection of mecholyl and eserine. Evidence that esterase and lipase can be separately determined in serum was provided by experiments with specific accelerators and inhibitors of enzymatic activity in serum, liver, and pancreas.

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# LIVER REGENERATION IN THE PRESENCE OF COMMON BILE DUCT OBSTRUCTION<sup>1</sup>

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HIGGINS and Anderson (1) working with rats and Mann, Fishback, Gay and Green (2), with dogs concluded that little or no parenchymal liver regeneration occurred when biliary flow was obstructed. As a result of the following experiments, we believe that liver regeneration does occur in the rat in the early mid-stages of biliary stasis to a degree equal to that occurring in pair-fed unobstructed controls. This is based on observations of liver mass (both wet and dry), liver protein and mitotic activity.

## METHODS

Male Wistar rats, averaging 250 grams in body weight, were placed on a synthetic non-protein diet (G-2) for 14 days. The animals, under ether anesthesia, were subjected to a 70 per cent partial hepatectomy, followed immediately by high ligation and division of the common bile duct. Animals partially hepatectomized only served as controls.

Postoperatively, four groups of biliary obstructed rats were fed a 10 per cent casein, low fat diet (G-6) while control groups were fed the same diet, some *ad libitum*, and others limited to the amount eaten by the obstructed animals. The rats were killed on the 2nd, 4th, 8th and 14th postoperative days under intra-peritoneal sodium amytal anesthesia. At both operation and autopsy, liver mass (both in the wet and dry state) was determined, and analyses for protein, glycogen and lipid were carried out as previously described (3). At autopsy, blood was obtained from the inferior vena cava for serum protein, prothrombin and icterus index determinations. All livers were examined histologically. Other groups of rats subjected to the same preoperative and operative procedures as those described were fed postoperatively, 1) a non-protein, low fat diet (G-2); 2) a high protein, low fat diet (G-1); and 3) a high fat (30%), 13.2 per cent protein diet (F-4) with and without supplemental desiccated pig bile<sup>4</sup> (0.5%). These rats were killed on the 14th postoperative day. The composition of all diets is given in table 1.

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<sup>4</sup> Desicol (Parke, Davis & Co.).

One group of rats after 14 days of protein depletion was subjected to high ligation and division of the common bile duct alone, without partial hepatectomy. They were fed diet G-6 and were killed on the 14th postoperative day. Control rats, pair-fed to these, had laparotomy only.

One further group of rats after 7 days' protein depletion had ligation of the common bile duct and after a second 7-day period of protein depletion were subjected to partial hepatectomy.<sup>5</sup> They were then fed a 10 per cent casein low fat diet (G-6) and were killed on the 14th day following the second operation. Parenteral vitamin K (Synkovite 1 mg.) was administered to each of these rats on three occasions near the time of their second operation.

Food consumption and body weight of all rats were recorded daily. Four days prior to operation and during the entire postoperative period, nitrogen analyses of feces and urine were done daily (4).

TABLE 1

	G-2	G-6	G-1	F-4
	%	%	%	%
Sucrose.....	91	81	73	48.9
Casein.....	0	10	18	13.2
Cod liver oil.....	3	3	3	3
Salts.....	4	4	4	5.3
CellufLOUR.....	2	2	2	2.6
Mazola.....				2
Crisco.....				25
Cal/gm.....	3.9	3.9	3.9	5.2

*Composition of Diets.* All diets contained a vitamin supplement consisting of the following quantities per 10.0 gm. unit of food: thiamine, riboflavin and pyridoxine, 0.1 mg. each; nicotinic acid, 1.0 mg.; inositol, 6.0 mg.; para-aminobenzoic acid 2.0 mg.; calcium pantethenate 0.6 mg.; and choline chloride 20.0 mg. In diet F-4, the amount of choline was doubled. All biliary obstructed animals received also oral vitamin K (Menadione) 0.05 mg/10 gm. of food.

## RESULTS

Essential data are summarized in table 2. Comparison of the 10 per cent casein-fed (G-6) biliary obstructed and control rats, killed on the 2nd, 4th, 8th and 14th postoperative days (*exper. 1*), is facilitated by the diagrams shown as figures 1 and 2.

Figure 1 shows the amounts of liver protein regeneration. All biliary obstructed animals suffered approximately a 50 per cent reduction of appetite, and, accordingly, some controls were pair-fed (limited) with the jaundiced rats, and others were fed *ad libitum*. It is seen throughout all postoperative periods that the biliary obstructed rats regenerated more liver protein than did unobstructed rats, whether pair-fed, or fed *ad libitum*.

A portion, however, of the protein regenerated in obstructed rats was due to

<sup>5</sup> From other studies, the median and left lateral lobes comprised 65 per cent (not 70%) of the total liver mass when biliary outflow was obstructed for this period.

TABLE 2. DATA OF BILIARY OBSTRUCTED AND UNOBSTRUCTED CONTROL RATS

EXPER. NO.	POSTOP. PERIOD	DIET	OPERATION	NO. OF RATS	INITIAL BODY WT.	POSTOP. WT. GAIN OR LOSS	LIVER REGENERATION <sup>1</sup>			NITRO-GEN <sup>1</sup>		PROTEIN	SE- RUM Prothrombin	ICTERUS INDEX	MITOSES/1000 LIVER NUCLEI
							Wet mass	Dry mass	Protein	Intake	Balance				
					gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm. %	%		
1A	2	G-6 10% Casein	Part-hep. C.B.D. ligat.	6	251 ±11	-9 ±3	0.71 ±.18	0.14 ±.01	0.13 ±.01	0.04 ±.01	-0.03 ±.02	4.66	80		10.3 ±2.1
	2 <sup>2</sup>	Non-protein	Part-hep.	5	242 ±11	-8 ±2	0.68 ±.10	0.19 ±.01	0.10 ±.01		0	4.43			10.9 ±4.0
1B	4	G-6 10% Casein	Part-hep. C.B.D. ligat.	6	255 ±13	-9 ±7	1.20 ±.13	0.26 ±.01	0.23 ±.01	0.09 ±.01	-0.04 ±.01	5.01	84		11.7 ±3.0
	4	G-6 Limited 10% Casein	Part-hep.	5	247 ±7	-9 ±2	0.65 ±.07	0.14 ±.02	0.20 ±.01	0.09 ±.01	-0.04 ±.01	4.46	100		1.0 ±1.2
	4 <sup>2</sup>	G-2 Non-Protein	Part-hep.	5	247 ±11	-7 ±4	0.99 ±.16	0.27 ±.10	0.14 ±.01		0	4.64			
	8	G-6 10% Casein	Part-hep. C.B.D. ligat.	5	251 ±10	-7 ±6	1.86 ±.42	0.39 ±.14	0.32 ±.04	0.24 ±.04	-0.06 ±.08	5.33	98	36	2.8 ±1.4
1C	8	G-6 Limited 10% Casein	Part-hep.	6	247 ±11	-15 ±2	0.78 ±.14	0.21 ±.03	0.23 ±.02	0.24 ±.01	-0.05 ±.02	5.38	95		0.2
	8	G-6 <i>Ad lib.</i> 10% Casein	Part-hep.	6	272 ±6	+7 ±5	1.46 ±.17	0.39 ±.07	0.26 ±.01	0.40 ±.04	+1.10 ±.03	5.29	93		0.6
	14	G-6 10% Casein	Part-hep. C.B.D. ligat.	6	259 ±24	-7 ±8	2.12 ±.23	0.45 ±.07	0.36 ±.04	0.46 ±.08	-0.02 ±.06	5.78	82	51	2.5 ±1.9
1D	14	G-6 Limited 10% Casein	Part-hep.	5	261 ±15	-12 ±3	0.79 ±.08	0.22 ±.07	0.25 ±.02	0.46 ±.03	.01 ±.03	5.53	100	19	0.4
	14	G-6 <i>Ad lib.</i> 10% Casein	Part-hep.	5	245 ±10	+34 ±9	2.01 ±.16	0.60 ±.05	0.34 ±.03	0.98 ±.13	.27 ±.05	5.19			
	14	G-2 Non-Protein	Part-hep. C.B.D. ligat.	8	262 ±18	-27 ±11	1.50 ±.22	0.30 ±.03	0.23 ±.02		-0.33 ±.06	4.88	70	36	
2	14	G-1 18% Casein	Part-hep. C.B.D. ligat.	9	246 ±21	+17 ±15	2.87 ±.44	0.63 ±.12	0.52 ±.08	1.09 ±.24	+0.33 ±.16	6.07	93	49	
	14	F-4 30% Fat	Part-hep. C.B.D. ligat.	4	260 ±10	30 ±11	2.10 ±.29	0.43 ±.06	0.37 ±.03	0.32 ±.07	-0.26 ±.04	5.58	70	51	
3	14	F-4 + Bile 30% Fat	Part-hep. C.B.D. ligat.	4	253 ±11	-33 ±11	2.14 ±.25	0.41 ±.05	0.36 ±.03	0.34 ±.12	-0.31 ±.08	5.70	76	50	
	14	G-6 10% Casein	C.B.D. ligat. 1 week Part-hep.	3	255 ±15	+17 ±17	1.99 ±.43	0.50 ±.10	0.31 ±.03	0.68 ±.16	+1.18 ±.13	5.61	73	35	
5	14	G-6 10% Casein	C.B.D. ligat.	6	253 ±5	+6 ±5	4.71 ±.32	1.06 ±.08	0.71 ±.05	0.64 ±.11	+0.09 ±.09	6.46	76	62	
	14	G-6 Limited 10% Casein	Laparotomy	5	253 ±9	+5 ±4	2.32 ±.09	0.68 ±.03	0.50 ±.02	0.62 ±.02	+1.10 ±.03	5.97	94		

<sup>1</sup> Liver regeneration and nitrogen intake and balances are expressed in gm/100 gm. initial body weight. Values shown are averages for each experimental group. Where individual values were determined, the standard deviation is shown: S.D. =  $\sqrt{\frac{\sum (\Delta x^2)}{n-1}}$

<sup>2</sup> Data previously published as *experiment 2A* and *3B* respectively, by Gurd, Vars and Ravdin (3).

bile duct proliferation and fibrosis, which was always observed. Some, too, might be associated with the increased fluid content of these livers either as plasma or lymph. In figure 1, the shaded non-outlined segment of the bars represents an estimation of

this non-parenchymal protein. It is seen that even with the subtraction of this portion, liver protein regeneration in the biliary obstructed livers occurs apace of the pair-fed controls but lags behind those fed *ad libitum*. No accurate method of calculating this portion of protein was found. The estimation of the extra fibrous tissue and duct

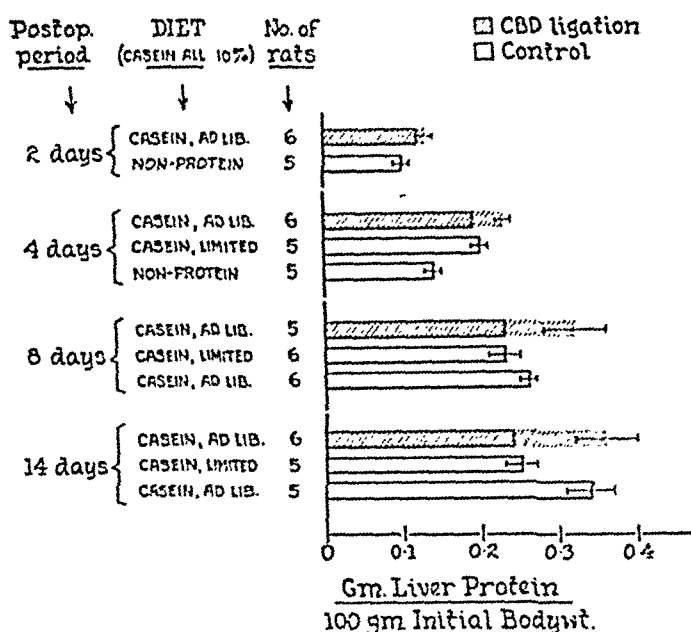


Fig. 1. LIVER PROTEIN REGENERATION in biliary obstructed and control rats following partial hepatectomy.

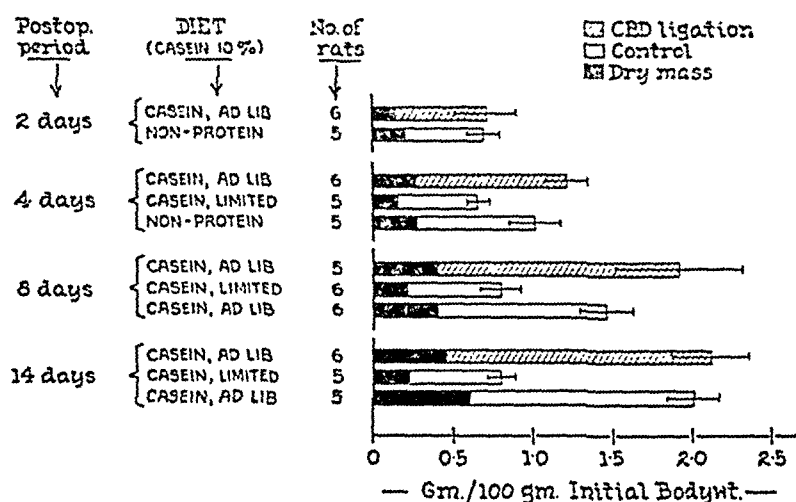


Fig. 2. LIVER MASS REGENERATION in biliary obstructed and control rats following partial hepatectomy.

protein was made from the histological appearance of the livers (Masson's trichrome, and Laidlaw's reticulum stain), while the extra fluid present in the biliary obstructed livers was considered to contain at most 8 per cent protein.

All biliary obstructed rats and pair-fed controls were in negative total nitrogen balance to an equal degree, while *ad libitum*-fed controls maintained a positive balance. Jaundice per se did not significantly increase the degree of negative nitrogen

balance over pair-fed non-jaundiced controls, when both had been previously protein depleted. This was not the case, however, when both groups had been well fed previously.

Figure 2 shows the amounts of liver mass regeneration (both wet and dry) occurring in these groups of rats. It is evident that, by weight as well as by protein, the biliary obstructed rats regenerated more than did both pair-fed and *ad libitum* fed controls.

Mitotic cell counts were made on sections of the livers from these groups of rats by the method used by Brues and Marble (5), that is the number of mitotic nuclei per 1000 hepatic cell nuclei. These counts were made under oil immersion, and approximately 20 fields dispersed throughout all parts of each section were required to be examined in order to count 1000 liver cells. Duct-cell mitoses were distinguished and excluded. Figure 3 graphically demonstrates mitotic activity in both biliary obstructed and unobstructed livers from groups of rats fed comparable diets. Mitotic

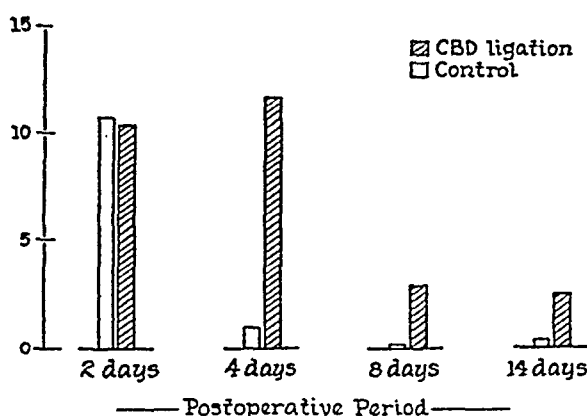


Fig. 3. NUMBER OF MITOSES PER 1000 HEPATIC CELL NUCLEI in biliary obstructed and control rats following partial hepatectomy.

counts of parenchymal cells in biliary obstructed livers and unobstructed controls were equal at the 2nd postoperative day. From that point on, mitotic activity in the controls rapidly decreased until mitoses were rare on the 8th and 14th postoperative days. In the obstructed livers, however, mitotic activity reached a peak on the 4th postoperative day and from then on decreased, but remained much higher than did that of the controls. It may be that the continued insult to the obstructed livers of the jaundice, and the increasing biliary distention, stimulated the parenchymal cells to greater regenerative efforts.

The biliary obstructed rats, which were fed various diets and killed 14 days postoperatively (*exper. 2*) regenerated liver protein in proportion to the amount of protein in the diet. With non-protein (G-2), 10 per cent casein (G-6), and 18 per cent casein (G-1) diets, liver protein regeneration was 0.23, 0.36 and 0.52 gm/100 gm. of initial body weight respectively. Vars and Gurd (3, 4, 6) obtained similar but lower results in unobstructed rats.

Two groups of biliary obstructed and partially hepatectomized rats (*exper. 3*) were fed a high fat diet containing 13.2 per cent casein (F-4). One of these groups received desiccated pig bile (Desicol) 0.5 per cent in the diet. When killed, 14 days

postoperative, no significant difference was noted in these two groups either in liver mass, liver protein, dietary intake or nitrogen balance. They regenerated liver protein (0.36 gm/100 gm. of initial body weight) equal to that regenerated by rats fed a low fat diet, although their nitrogen and caloric intake was less.<sup>6</sup> They were, however, in marked negative nitrogen balance and in poor physical condition. Total postoperative fecal fat determinations were made on the two groups fed a high fat diet.<sup>7</sup> A slight but greater fecal fat excretion occurred in the group fed bile, although the fat intake of the two groups was practically equal. Fecal nitrogen excretion was unaffected. It may be that the presence of bile in the diet stimulated intestinal motility, so that there was less time for fat absorption. Fecal fat excretion in biliary obstructed rats fed a 3 per cent fat diet compared to unobstructed pair-fed controls was found to be twice as great.<sup>8</sup>

One group of rats, having been protein-depleted for 14 days, then had their common bile ducts ligated, but without partial hepatectomy (*exper. 5*). They were then re-alimented on a 10 per cent casein low fat diet (G-6). A control group, subjected to laparotomy alone, was pair-fed with the above. When killed, on the 14th postoperative day, the biliary obstructed rats had livers which weighed wet  $4.71 \pm 0.39$  gm., dry  $1.06 \pm 0.08$  gm., and contained  $0.71 \pm 0.05$  gm. of protein, all per 100 gm. of initial body weight. Control livers weighed wet  $2.32 \pm 0.09$  gm., dry  $0.68 \pm 0.03$  gm., and contained  $0.50 \pm 0.02$  gm. of protein. The nitrogen intake and balance in both were identical. These two groups of rats were subjected to similar procedures in all respects, except for the addition of common bile duct ligation in one. Upon the assumption that their liver protein restitution should have been the same, the difference in liver protein between the biliary obstructed livers (0.71 gm.) and the unobstructed controls (0.50 gm.) may be considered to be the protein of duct proliferation, fibrosis and edema. The factor .704 corrects for this portion of protein.

Using this factor to correct all 14-day common bile duct ligated and partially hepatectomized rats fed various diets, we may presume to obtain relatively true protein regeneration values of 0.16, 0.25, and 0.37 grams with non-protein (G-2), 10 per cent casein, (G-6) and 18 per cent casein (G-1) diets respectively. These values are identical to the protein regeneration which occurred in non-obstructed controls fed comparable diets.<sup>9</sup>

The group of rats subjected to common bile duct ligation, and one week later to partial-hepatectomy (*exper. 4*), fared better than expected. They ate well a 10 per cent casein, low fat diet (G-6), and were killed on the 14th day after partial hepatectomy.

<sup>6</sup> Biliary obstructed rats fed 30 per cent fat diet had N intake = .33 gm; cal. intake = 101 cal.; cal. intake less fecal fat-excretion calcs. = 78 cal.; N balance = -.29 gm. Biliary obstructed rats fed 3 per cent fat diet had N intake = .46 gm.; cal. intake = 139 cal.; and N balance = -.02 gm. (all per 100 gm. initial body weight).

<sup>7</sup> Biliary obstructed rats fed 30 per cent fat diet with and without bile had fat intakes of 5.6 and 5.3 gm., and fecal fat excretions of 3.11 and 1.95 gm. respectively per 100 gm. initial body weight.

<sup>8</sup> Biliary obstructed rats fed 3 per cent fat diet had a fat intake of 1.0 gm., and fecal fat excretion of 0.34 gm., while unobstructed pair-fed controls had fecal fat excretion of 0.14 gm. per 100 gm. of initial body weight.

<sup>9</sup> Control values for diets G-2 and G-1 of  $0.17 \pm 0.01$  and  $0.37 \pm 0.04$  gm/100 gm. of initial body weight were obtained from data of Vars and Gurd (4).

They regenerated liver mass wet,  $1.99 \pm 0.43$  gm., dry  $0.50 \pm 0.10$  gm., and liver protein  $0.31 \pm 0.03$  gm., with a nitrogen intake of  $0.68 \pm 0.16$  gm., and a positive nitrogen balance of  $0.18 \pm 0.13$  gm. Comparable unobstructed rats (4) regenerated liver mass wet,  $1.98 \pm 0.38$  gm., dry  $0.60 \pm 0.13$  gm., and liver protein  $0.30 \pm 0.02$  gm., with a slightly greater nitrogen intake of  $0.77 \pm 0.09$  gm., and a balance of  $+0.24 \pm 0.06$  gm. all per 100 grams of initial body weight. These previously biliary obstructed rats also regenerated liver space with non-obstructed rats.

Lipid and glycogen determinations were done on all pooled livers of groups of rats at both operation and death. Lipid values at operation were constant at 6 to 7 per cent. At death they all remained the same or were less (3-4%). No livers were fatty in either the biliary obstructed or unobstructed rats, even when the diet contained 30 per cent fat. Glycogen values at operation varied from 6 to 10 per cent and at death were universally below 1 per cent in obstructed animals and in pair-fed controls, but were maintained around 6 per cent in *ad libitum*-fed controls.

Plasma proteins at death were all of a fairly uniform level of 5 gm. per cent, although rats which received higher protein diets tended to have slightly higher plasma protein values than did rats with a lesser protein intake.

Prothrombin percentage never reached critical levels in any of the rats. Biliary obstructed rats had levels of approximately 70 to 80 per cent while controls had 90 to 100 per cent. The icterus index of the jaundiced rats varied from 35 to 62 (normal 18).

Seventy-eight rats were subjected to common bile duct ligation with and without partial hepatectomy. Of these 14 could not be used either because of some associated pathology or death. The over-all mortality of the biliary obstructed animals was 15.4 per cent.

Grossly, all biliary obstructed livers developed increasing fibrosis depending on the duration of the obstruction. At 14 days, the livers were large, tawny in color and of a firm rubbery consistency. Several showed early fine nodularity, and pin point areas of necrosis were observed. The common bile ducts were tense, thin-walled and markedly dilated, containing from one to three cc. of usually pale watery bile, but occasionally thicker turbid bile. Adhesions were frequent, and often the duodenum was displaced by the distended choledochus, but no instances of intestinal obstruction were noted. Routinely the intestinal tract was inspected to ascertain the absence of bile staining. Occasionally in rats that died and in a few which were killed a massive lobar necrosis was observed in the livers, particularly where the distended common bile duct caused pressure of the liver against the rib margin or vertebral column. This distribution of necrosis was previously observed and reported by Estrada, Simpson and Vars (7) in their studies of gastric distention and liver damage. Data obtained from these livers were not included in the computation of the results. Two or three livers contained small hilar abscesses, invariably accompanied by wound infection. These also were excluded. No other organs of the body showed any abnormality, although in long standing biliary obstructed rats, the spleen always appeared slightly larger than in controls. Ascites was not observed.

Microscopically, the biliary obstructed livers showed a progressive inter and intralobular fibrosis, occurring predominantly in portal areas. Marked ductal pro-

liferation was present. The liver cells showed no evidence of fatty infiltration. They were frequently irregular with shrunken cytoplasm, no doubt partly due to their low glycogen content. Usually they stained pale, except for occasional cords of cells which stained darker. Tiny focal areas of necrosis were frequently observed in various stages of formation and repair. Small numbers of inflammatory cells were seen in these areas of necrosis but not elsewhere. The kidneys and spleen were also examined microscopically and showed no marked abnormality. Bile thrombi were seen in the kidneys; the spleens were congested and appeared to have increased fibrous tissue stroma.

#### CONCLUSIONS AND SUMMARY

Protein-depleted rats, subjected to partial hepatectomy and to simultaneous high ligation and division of the common bile duct, regenerated in the 14-day post-operative period liver mass and liver protein in excess of *ad libitum*-fed and pair-fed control rats subjected to the same procedures except for ligation and division of the common bile duct. After subtracting the amount of (liver) protein estimated to be due to bile duct proliferation and hepatic fibrosis, the parenchymal cell protein regeneration in biliary obstructed rats equalled that occurring in unobstructed pair-fed controls. Liver protein regeneration in biliary obstructed rats, as in unobstructed rats, was proportional to the protein intake in the post-operative period.

A high fat diet did not decrease liver protein production though it increased the negative nitrogen balance. The addition of bile to the high fat diet caused no material change in metabolism or liver regeneration. Jaundice per se in previously protein-depleted rats did not alter the nitrogen balance from that of unobstructed pair-fed rats. Counts of parenchymal cell mitoses at intervals following partial hepatectomy indicated active parenchymal regenerative efforts on the part of the biliary obstructed livers.

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# EFFECT OF ECK FISTULA FORMATION, SIMPLE PORTAL OBSTRUCTION AND 'MEAT INTOXICATION' ON SERUM PHOSPHATASE AND DYE CLEARANCE OF ADULT DOGS

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THE general effects of depriving the liver of its portal blood supply by means of an Eck fistula (portal vein ligation proximal to the site of portacaval anastomosis) has been described by numerous investigators (1-3). It is commonly agreed that Eck fistula dogs ordinarily manifest anorexia and weight loss, frequently dying in an advanced state of cachexia, anemia and debility. Impairment in the ability of the Eck fistula dog to synthesize plasma proteins, hemoglobin (4) and bile salts (5), to store fluids (6) and destroy uric acid (7) have been reported. However, dye clearance studies reported by Bollman and Mann (7) failed to demonstrate definite impairment in liver function, although their comments indicate that an impairment could be demonstrated with large amounts of rose bengal. Portal obstruction without portacaval anastomosis results in less atrophy of the liver than occurs in the Eck fistula dogs and the animals remain in relatively good health (3).

The intolerance of Eck fistula dogs to meat has long been recognized (1), but Whipple *et al.* have shown (4) that Eck fistula dogs can be maintained in relatively good health for several years. It has not been reported that dogs with simple portal obstruction are susceptible to meat intoxication.

The literature pertaining to Eck fistula and simple portal vein obstructed animals contains little data with regard to dye clearance and essentially no information concerning serum phosphatase activity. Previous studies from this laboratory have shown that the rose bengal dye clearance and serum phosphatase tests can be used to demonstrate changes in hepatic function under certain circumstances. In the present study these tests were applied to Eck fistula, simple portal vein obstructed, and normal dogs, with and without meat feeding.

## EXPERIMENTAL PROCEDURE

Medium to large (30-40 lb.) adult dogs of both sexes were used for this study. The postoperative diet routinely consisted of Pard, bread and milk. Initially the Eck fistula was formed according to the procedure described by Fishback (2). More recently a special clamp was devised to aid the formation of this anastomosis (8). Simple complete portal ligation was carried out in two stages. The second operation was usually 3 to 4 weeks after partial portal obstruction had been produced. Obstruction of the portal vein both with and without portacaval anastomosis was always just proximal to the point of entrance of the pancreatic vein into the main portal vessel. 'Meat intoxication' was produced by feeding 50 gm/kg. of ground raw lean horsemeat daily. Frequently it was necessary to force-feed the operated dogs, in which case the diet was more completely retained if fed in divided portions. The methods of estimation of serum phosphatase and rose bengal dye clearance have been described

previously (9). These tests were usually made at weekly intervals on each animal during the period of study, which varied from a few weeks to several months.

## RESULTS

The effects of Eck fistula formation on the dye clearance and serum phosphatase of the dog are shown in the accompanying table. Without exception there was a definite decline in the dye clearance. This decline was apparent soon after the operation and usually progressed, reaching values in some instances that were only approximately one-third of the preoperative value. Following Eck fistula formation the serum phosphatase underwent a definite increase (see table). In some animals it reached values that were many times normal, while in other instances the rise was not so striking. Usually there was an inverse relation between the dye clearance and the serum phosphatase value.

Simple portal obstruction usually produced a slight decline in the dye clearance, accompanied by an increase in serum phosphatase (see table). The change from the normal values for these tests after simple portal obstruction was less striking than that after Eck fistula formation. The *t*-ratios indicate that the changes are significant after both operations.

'Meat intoxication' was produced in 11 Eck fistula dogs. The incidence of meat intoxication was increased by using the clamp in preparing the portacaval anastomosis. In every instance the appearance of the symptoms of 'meat intoxication' (ataxia, blindness, spasticity) was associated in Eck fistula dogs with an increase in serum phosphatase (see table). The individual increase varied considerably and the striking increase in one animal from 11 to 65 Bodansky Units lowered the *t*-ratio. Including this animal it is 2.54; excluding this animal, 3.5. However, both values indicate that there is little likelihood that the observed differences could be explained as due to chance variation (2% and 1%, respectively, 9a). In one instance a marked increase (88 units in 26 days) in serum phosphatase occurred in an Eck fistula dog during the period of meat feeding, but the dog remained in good health. In 7 out of 12 instances of 'meat intoxication' in Eck fistula dogs there was a striking associated decline in dye clearance, while in 5 other instances this change was relatively slight. The onset of 'meat intoxication' was often sudden; some dogs became moribund in 24 to 48 hours after the onset of symptoms. The average time required for 'meat intoxication' to occur was 9 days after the onset of meat feeding. The animals that rapidly became moribund showed the least change in liver function tests.

There were no consistent changes in the liver function tests during the time that the portal vein obstructed dogs were fed meat (see table). 'Meat intoxication' was produced in one of 5 simple portal vein obstructed dogs fed exclusively on horsemeat without significant changes occurring in the liver function tests. This animal manifested the classical symptoms of 'meat intoxication' after 7 days on meat and was twice presumed to be dying. However, it made a spontaneous recovery, resumed eating meat, and did not develop meat intoxication again even though the daily intake of meat was increased to 100 gm/kg. daily for 3 weeks.

Four normal dogs fed meat showed no change or a slight decline in dye clearance. Their phosphatase values remained within the normal range (see table).

TABLE I

CONDITION OF DOGS	DYE CLEARANCE <sup>1</sup>							SERUM PHOSPHATASE <sup>2</sup>							Exper. period	Spread in days
	No. of dogs	Aver.	S.D.	S.E.	Diff. of means	S.E. of diff.	t ratio	No. of dogs	Aver.	S.D.	S.E.	Diff. of means	S.E. of diff.	t ratio		
Eck fistula Before operation After operation	24	106	10.9	2.21	61	3.4	18	22	2.3	1.2	.256	10.2	1.71	5.96	31 <sup>3</sup>	9-69
	24	45	15.8	3.24				7	12.5	7.84	1.67		7			
Portal obstruction Before ligation After ligation	6	114	13.3	5.36	34	7.4	4.6	7	1.8	1.10	0.41	4.6	1.25	3.71	36 <sup>4</sup>	40-119
	7	80	13.3	6.01				7	6.4	3.1	1.17		7			
Eck fistula Before meat intoxication During meat intoxication	12	51	11.4	3.31	17	4.23	4.03	12	6.6	3.0	.87	12.2	4.86	2.54	9 <sup>5</sup>	3-31
	12	34	9.38	2.7				12	18.8	16.0	4.65		12			
Portal obstruction Before meat diet During meat diet	5	87	15.9	7.1	11	12.9	.85	5	4.9	1.24	.55	1.6	2.48	.645	69 <sup>4</sup>	34-144 <sup>4</sup>
	5	76	33.1	14.9				5	6.5	5.47	2.45		5			
Normal Before meat diet During meat diet	4	111	11.5	5.75	14	9.74	1.45	4	1.75	1.17	.59	.57	1.02	.56	15 <sup>6</sup>	7-20 <sup>6</sup>
	4	97	14.7	7.35				4	1.75	1.17	.59		4			

<sup>1</sup> = arbitrary units; <sup>2</sup> = Bodansky units; <sup>3</sup> = days after Eck fistula operation; <sup>4</sup> = days after portal ligation; <sup>5</sup> = days required for meat intoxication to develop in Eck fistula dogs; <sup>6</sup> = days on meat diet.

## DISCUSSION

The observations herein presented indicate a definite reduction in the rate of rose bengal excretion as a result of Eck fistula formation. The reduction was variable in amount, but the average reduction was to a value approaching half of the preoperative one. The lowest values for dye excretion were usually not obtained immediately after operation; therefore, it is reasonable to suppose that secondary changes occurring in the liver contribute to the loss of excretory function for the dye. The liver is reduced in size after Eck fistula formation, indicating that considerable parenchyma must be lost. In addition, an increase in liver fat may occur. Failure of others to demonstrate clearly the effect of Eck fistula formation on dye clearance was probably due to a difference in the technique used for demonstrating the rate of dye disappearance.

Simple portal obstruction also caused a definite reduction in the rose bengal dye clearance. However, the effect was less marked and less consistent than the reduction caused by Eck fistula formation. The difference between the two groups probably reflects the difference in the degree of reduction in hepatic circulation. Portal hypertension is produced by simple ligation of the vessels and this stimulates the development of a collateral circulation, part of which goes to the liver, as evidenced in the group herein presented by the enlargement of numerous small vessels entering the hilus of the liver. Formation of an Eck fistula with an adequate stoma does not lead to portal hypertension, as judged in this group by the absence of well-defined venous collaterals at the hilus of the liver. The fact that there is less atrophy of the liver in dogs with simple portal obstruction than in Eck fistula animals is further evidence that the former group has less reduction in venous blood entering the liver.

'Meat intoxication' in the Eck fistula dog frequently caused a further reduction in dye clearance. The average effect in the 12 instances reported was less definite than the results obtained in certain individual cases. In other instances 'meat intoxication' was not associated with any further decrease in the dye clearance. The results show that in slightly over half of the instances 'meat intoxication' produced a definite and rapid decline in the rate of removal of rose bengal from the circulation. This observation does not agree with that recently reported by Drill (10). Meat feeding in the simple portal vein obstructed dogs caused a significant decrease in dye clearance in only 2 animals.

An increase in serum phosphatase always resulted from Eck fistula formation. The magnitude of increase and the rate of change varied from animal to animal, but the tendency was the same in every instance. Probably there is a relation between the size and patency of the stoma and the postoperative rise in serum phosphatase activity. The serum phosphatase rise reached a maximum at varying times after Eck fistula formation and was frequently but not invariably associated with a minimum dye clearance in a particular animal. 'Meat intoxication' consistently caused a further increase in serum phosphatase in the Eck fistula dog. In some animals the inverse relation between the serum phosphatase and dye clearance (previously demonstrated in the protein-deficient dog, 9, 11), was apparent, whereas meat poisoning caused a definite change in serum phosphatase with little or no further decline in dye clearance in other animals. There are, however, interesting exceptions to both of these statements. For instance, the dog with simple portal obstruction that developed meat intoxication showed a slight decline in serum phosphatase at the time of 'meat intoxi-

cation.' An Eck fistula dog, which remained in good health during 26 days of meat feeding, showed a marked rise in serum phosphatase during this period with subsequent decline when the meat diet was discontinued.

There is a difference of opinion as to the significance of the increase in serum phosphatase that occurs in relation to liver disease. Some (12, 13) hold that the rise is the result of impaired excretion of the enzyme from the blood stream by the liver, whereas others (14, 15) maintain that the increase results from the liver phosphatase gaining access to the circulation in increased amounts. If the rise in serum phosphatase were simply due to a failure of hepatic excretion of phosphatase from the blood stream, its rise would always inversely parallel changes in dye clearance. There are numerous instances wherein this association does not seem to exist. It was also observed by Dameron and the author (16) that bile or jaundiced blood rich in phosphatase injected intravenously into bile fistula dogs failed to alter the phosphatase output in the bile, although the injected phosphatase disappeared from the circulation. In a previous publication (15) other experimental evidence was presented favoring the view that the increased serum phosphatase resulting from obstruction or injury to the liver largely originates within the liver itself rather than in the skeleton or elsewhere in the body.

Regardless of the origin of the serum phosphatase, its rise in the absence of extra-hepatic bile duct obstruction indicates that Eck fistula formation causes active damage to the liver and that this damage is aggravated by 'meat intoxication.' The fact that 'meat intoxication' contributes to disturbed hepatic function as measured by dye clearance and serum phosphatase may be taken as evidence that further failure of hepatic function is usually associated with 'meat intoxication' in the Eck fistula dog. The occurrence of 'meat intoxication' without changes in dye clearance or serum phosphatase activity in a dog with simple obstruction of the portal vein indicates that neither of these measurements is directly related to the hepatic failure that caused these symptoms.

#### SUMMARY

Eck fistula formation and simple portal vein obstruction have qualitatively the same effects upon serum phosphatase and rose bengal dye clearance. Eck fistula formation consistently reduced the rose bengal dye clearance and increased the serum phosphatase of adult dogs. 'Meat intoxication' regularly caused a further increase in the serum phosphatase and frequently caused a definite decline in the dye clearance of Eck fistula animals. However, meat feeding did not consistently produce any further change in the liver function tests of the simple portal vein obstructed dogs, although 'meat intoxication' was produced in one such animal. The foregoing evidence indicates that neither liver function test is an index of the hepatic failure that results in the symptoms of 'meat intoxication.'

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# EFFECT OF IMPAIRED HEPATIC CIRCULATION ON PLASMA FREE AMINO ACIDS OF DOGS

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**E**CK fistula dogs fed a diet of raw horsemeat and water frequently exhibit signs of 'meat intoxication.' These include muscular rigidity similar to that seen in decerebrate animals and loss of senses, particularly vision. Some animals appear semi-stuporous, with ataxic gait and loss of righting reflexes. Animals with portal obstruction generally do not intoxicate upon the raw horsemeat diet. The literature contains no information concerning the effect of meat intoxication on the plasma concentration of the individual amino acids.

Early investigations by Van Slyke (1) indicated that during digestion there was a greater fall in the amino nitrogen content of the blood in its passage through the liver of the normal animal than through the entire remainder of the body. The reduction of blood supply to the liver by Eck fistula formation did not increase the blood amino nitrogen content of dogs, according to Whipple and Van Slyke (2). Acceleration of amino acid liberation in their animals by meat feeding or toxic tissue autolysis failed to elevate amino nitrogen levels.

The advent of microbiological procedures for the determination of individual amino acids has made possible the detailed study of these substances in animals with altered hepatic circulation. This report concerns the effect of Eck fistula formation on the plasma free amino acids of fasted dogs and the effects of meat feeding on dogs with portal obstructions and those with Eck fistulas.

## EXPERIMENTAL

*Surgical Procedures.* Initially Eck fistulas were formed according to the procedure described by Fishback (3). More recently a special clamp was devised to aid the formation of this anastomosis (4). Simple complete portal ligation was carried out in two stages. The second operation was performed 3 to 4 weeks after partial portal obstruction had been produced by constriction of the portal vein to approximately half of its normal diameter just proximal to its junction with the pancreatic vein.

*Diets.* Dogs which were used for the determinations of normal ranges of amino acids were maintained on bread, cooked horsemeat and water. Animals with Eck fistulas were fed a bread-milk-Karo syrup ration. The meat diet for the production of intoxication consisted of raw horsemeat, 50 mg/kg. body weight per day.

*Microbiological Determinations.* Heparinized blood samples were centrifuged and the plasma treated according to the method of Hier and Bergeim (5). *L. arabinosus* 17-5 was used for the assay of tryptophane, leucine, isoleucine and valine. Histidine,

methionine, tyrosine, proline, arginine, glycine, cystine, serine and lysine were measured with *Leuconostoc mesenteroides* P-60. *Streptococcus faecalis* R. was employed in the assay of threonine and *L. casei* 7469 for phenylalanine. Assay media were dis-

TABLE 1. FASTING LEVELS OF PLASMA FREE AMINO ACIDS IN NORMAL DOGS

AMINO ACID	NO. ANIMALS TESTED	NORMAL RANGE	AVERAGE	AMINO ACID	NO. ANIMALS TESTED	NORMAL RANGE	AVERAGE
<i>μg/ml. plasma</i>				<i>μg/ml. plasma</i>			
Histidine.....	36	6.6-39.7	22.34	Arginine.....	12	6.0-52.5	24.9
Tryptophane.....	22	3.2-26.0	13.25	Threonine.....	21	11.4-48.3	25.9
Methionine.....	27	2.2-18.0	9.7	Phenylalanine...	13	8.2-22.5	14.5
Tyrosine.....	28	4.9-33.0	18.4	Cystine.....	13	9.1-23.3	16.4
Proline.....	17	7.5-34.2	17.2	Serine.....	13	4.8-18.6	11.3
Leucine.....	13	16.2-45.0	34.5	Glycine.....	10	17.8-39.5	27.2
Isoleucine.....	12	2.4-22.5	13.4	Lysine.....	7	22.5-41.6	30.8
Valine.....	15	15.0-37.5	27.2	Aspartic acid....	20	< 1	< 1

TABLE 2. CONSTANCY OF FASTING LEVELS OF FREE AMINO ACIDS IN DOG PLASMA

WEEKLY SAMPLES	ISOLEUCINE	LEUCINE	METHIONINE	TRYPTOPHANE	TYROSINE	VALINE
	<i>μg/ml. plasma</i>					
<i>Dog 189</i>						
A	23.2	24.3	6.9	7.1	7.8	13.6
B	29.6	28.2	5.4	6.3	6.0	18.6
C	39.7	26.3	7.2	8.7	6.3	20.3
D	33.5	28.0	7.8	8.4	12.6	24.6
<i>Dog 186</i>						
A	37.8	23.6	7.9	6.5	3.9	22.5
B	36.0	19.4	7.8	7.1	4.5	19.5
C	22.8	17.4	6.6	9.3	3.6	20.4
D	30.8	22.5	7.8	8.3	6.3	22.7
<i>Dog 185</i>						
A	41.7	30.0	7.8	7.4	10.5	20.4
B		31.8	6.6	8.6	9.3	22.5
C	36.3	30.7	6.0	10.8	6.3	21.9
D	28.4	30.7	7.2	10.7	10.2	22.8
<i>Dog 182</i>						
A		30.0	6.9	6.9	8.4	29.6
B		30.0	7.2	7.6	7.5	22.2
C		30.0	8.7	8.1	9.9	23.4
D		33.7	8.1	9.5		33.3

pensed in 1 ml. amounts, and samples and water brought the final volume in the tubes to 2 ml. Each sample was assayed in duplicate at 3 levels. Standard curves were set up in triplicate for each assay. All data are derived from plasma obtained 12 or more hours after the last feeding, with the exception of that contained in table 5.

## RESULTS

Table 1 provides a list of the amino acids studied in normal dogs. The ranges and averages obtained from this survey were employed for comparison with blood levels of dogs with hepatic insufficiency.

TABLE 3. FASTING LEVELS OF PLASMA FREE AMINO ACIDS IN DOGS WITH SIMPLE PORTAL OBSTRUCTIONS ON HORSEMEAT INTOXICATION DIET

DOG NO.	DAY OF DIET	OBSERVED CONDITION	HISTIDINE	TRYPTOPHANE	METHIONINE	TYROSINE	PROLINE	VALINE	ARGININE	THREONINE	PHENYLALANINE
38	0	Normal	18.0		7.5	8.2	10.5			25.4	
	13	Normal	21.0		7.5	11.1	9.6			12.5	
53	0	Normal	7.5	8.0	6.7	13.5	4.8				
	5	Normal	30.0	9.6	13.5	22.0	15.0				
	12	Normal	16.5	4.8	3.9	13.8	6.6				
50	0	Normal	15.6	9.3	7.5	35.1	12.3			45.0	
	5	Normal	42.9 <sup>1</sup>	12.0	14.2	36.6	22.2			37.5	
	7	Sick	45.6 <sup>1</sup>	10.8	21.7	41.1	30.6			48.7	
	12 <sup>2</sup>	Very sick	24.0	7.5	4.2	18.0	0.0 <sup>1</sup>			28.0	
	20	Improved	18.0	7.1	6.5	15.0	9.0			17.5	
	24	Improved		9.9	14.7	21.0					
	28	Improved		3.0	13.6	12.0					
55	0	Normal		6.8	9.7	7.5		10.6	31.5		13.5
	6	Normal		7.5	12.7	11.4			60.0 <sup>1</sup>		24.4
	53	Normal		10.5	9.7	8.1			47.7		14.2
	62	Normal		14.3	9.6	12.0		30.0			18.4
	67	Normal		10.8	20.6	8.4		16.2			15.0
73	0	Normal	11.2	6.6		11.4	17.7		52.5		17.5
	6	Normal	9.0	8.1		11.4	16.5		37.5		12.7
	53	Normal				14.4	16.8		52.5		16.8
	62	Normal		15.0		11.4	10.9				12.5
	67	Normal		15.9		12.9	16.5				12.9
98	0	Normal		8.3	9.3	9.0		16.8	37.5		13.8
	6	Normal		9.0	15.0	9.0		17.8	43.5		10.7
	53	Normal		15.0	11.1	6.0		32.8	72.3 <sup>1</sup>		19.5
	62	Normal			9.9	7.5		30.0			16.8
	67	Normal		11.3	10.2	12.0		33.8			15.7

<sup>1</sup> Outside of normal range of plasma values for dogs.  
on 11th day.

<sup>2</sup> Intoxication too severe for feeding

To determine the constancy of fasting levels in a given animal, samples were obtained from 4 dogs during 4 consecutive weeks. Table 2 shows the degree of variation found in the levels of 6 amino acids. Methionine and tryptophane were the least variable; isoleucine, the most. The results of this experiment indicate that there is

TABLE 4. FASTING LEVELS OF PLASMA FREE AMINO ACIDS IN ECK FISTULA DOGS MAINTAINED ON HORSEMEAT INTOXICATION DIET

DOG NO.	DAY OF DIET	OBSERVED CONDITION	HISTIDINE	TRYPTOPHANE	METHIONINE	TYROSINE	PROLINE	ARGININE	THREONINE	PHENYLALANINE	CYSTINE	LEUCINE	ISO-LEUCINE	VALINE	LYSINE	GLYCINE	SERINE
										$\mu\text{g/ml. plasma}$							
62-I	0	Normal	24.6	14.4	7.5	14.2	19.2		20.7								
	11	Intoxicated	33.9	24.0	17.8	37.5	17.6		21.2								
62-II	0	Normal	18.3	10.0	6.0	30.3	12.3	33.3	46.8	15.7							
	5	Normal	37.5	10.8	14.5	37.5	23.1		36.5								
	10	Sick	37.5	12.0	17.5	37.6	21.5	51.9	41.2								
	20	Intoxicated		12.3	21.3 <sup>1</sup>	42.6 <sup>1</sup>		47.4	37.5	75.0 <sup>1</sup>							
51	0	Normal	25.0	10.5	12.0	16.4	25.0		25.8								
	28	Normal	26.0	12.3	11.1	24.3	15.0		15.3								
43	0	Normal	23.0	13.2	3.9	6.3			28.2								
	20	Normal	24.2	13.8	12.3	26.0			13.6								
61	0	Normal			13.9	38.7		38.0									
	8	Terminal			49.5 <sup>1</sup>	72.0 <sup>1</sup>		75.0 <sup>1</sup>									
85	0	Normal	18.6	10.1	11.2	28.2	19.8	6.0	34.5	17.1							
	6	Intoxicated	39.0	8.4	9.0	26.2	19.5	37.5	34.5	22.1							
74	0	Normal	22.2	19.5	14.4	39.6	22.5		33.7								
	2	Intoxicated	54.0 <sup>1</sup>	10.8	26.7 <sup>1</sup>	48.9 <sup>1</sup>	30.0		61.9 <sup>1</sup>								
76	0	Normal	11.1		27.9	27.3	9.9	37.5	20.4	9.2							
	5	Sick	24.9	7.8	8.5	27.0	13.8	30.5	28.0	12.0							
	7	Terminal	31.5	8.1	15.7	31.5	16.2		33.7	22.8							

I12	0	Normal Sick Terminal		8.3 5.7 2.7	9.6 9.7 15.0	12.0 12.0 29.4	19.8 44.1 60.0 <sup>1</sup>	18.0 16.6 42.7 <sup>1</sup>											
2D	0	Normal	32.2																
	6	Terminal	22.8																
155	0	Normal																	
	5	Sick																	
159	0	Normal	18.9	4.1															
	7	Sick	11.4	4.3															
140	15	Terminal	36.3	6.7															
	0	Normal	42.6		12.4														
156	7	Normal			10.6														
	8	Terminal	42.3		22.8 <sup>1</sup>														
	0	Normal		9.2	7.2														
	3	Terminal		7.4	3.3														

<sup>1</sup> Outside the normal range of plasma values for dogs.

relatively little fluctuation from week to week in 5 of 6 plasma-free amino acids of normal dogs on a bread and cooked-meat diet. It was considered necessary to establish this point in order to evaluate the results on control animals.

Plasma free amino acids were measured in 5 dogs before and after Eck fistula formation. The post-operative samples were taken 24 to 30 days after surgery. All 5 animals showed a slightly reduced plasma cystine, while 4 of 5 had less leucine and 3 of 5 less serine after the operation. Arginine was slightly reduced in the 2 animals tested. Tryptophane exhibited little fluctuation. Histidine was elevated slightly in 2 of 3 dogs, remaining the same in the third animal. None of the elevated or depressed levels were outside the ranges established previously as normal.

Three normal dogs and 13 dogs with Eck fistulas were placed on a raw horsemeat diet. The normal dogs showed no signs of poisoning even after 21 days. Eleven of the 13 with Eck fistulas developed signs of meat intoxication 2 to 11 days after the beginning of meat feeding. Eight of the 11 died. *Dog 62* was employed in 2 successive experiments and developed severe intoxication each time.

Six dogs with simple obstructions were included in the feeding experiments. Only one dog of this group became ill. He was noticeably sick on the 6th day of diet and on the 7th day appeared moribund. Meat was continued, by force when necessary, except on the 7th and 11th days when the animal was thought to be dying. The dog survived and resumed eating meat spontaneously, even when the ration was increased to 100 gm/kg. The normal dogs on the horsemeat diet showed decreased fasting levels of plasma free histidine, methionine, arginine, lysine and glycine. Tyrosine, isoleucine and serine remained fairly constant while tryptophane rose slightly in the one animal studied for this compound.

Tables 3 and 4 contain the results of amino acid assays on the plasma of the remaining dogs. They also show the length of time the animals had been on the raw horsemeat diet and their status at the time the blood samples were drawn. Two of the dogs with simple portal obstruction were found to have arginine levels above the upper limit of normal although they exhibited no sign of intoxication. *Dog 50*, which manifested the typical appearance of an intoxicated animal, showed a rise in amino acid levels at the time when poisoning became apparent, followed by a generalized reduction. In some cases the amino acids dropped below the levels obtained before meat feeding was begun. Proline was not detectable in this animal at the height of the disorder. Three of the 4 dogs studied for histidine showed elevated plasma levels. In the case of *dog 50* histidine was above normal.

*Dogs 51* and *43* had Eck fistulas but did not become intoxicated on the raw-meat diet. They showed a rise in tyrosine and a drop in threonine levels after 28 and 20 days, respectively, on the diet. The Eck fistula dogs which developed meat intoxication differed from the normal in that most of the amino acids were elevated. Six of 8 dogs showed increased histidine. Six of 7 had elevated tyrosine levels. Seven of 7 showed increased methionine, 4 of 5 increased leucine and 3 of 4 elevated glycine levels. Arginine rose in 4 out of 5 dogs. Cystine and phenylalanine increased in all dogs studied for these amino acids, and serine and proline rose in 3 of 5 animals. Valine, tryptophane, isoleucine, lysine and threonine gave inconsistent results. Six of the 11 intoxicated dogs had fasting plasma levels above normal for 2 to 5 amino acids.

Table 5 shows the rise in plasma free amino acids following a meal of raw horse-meat. These data are the only ones submitted which do not represent fasting levels. The differences between values before and after feeding are greater for the Eck fistula animals than for the normal in all cases except tryptophane. Seven amino acids in the intoxicated and 6 in the unintoxicated Eck dog were present after feeding in amounts higher than the upper limit of the normal fasting levels. Only tryptophane and valine were in excess in the control. Three amino acids in the normal dog and 5 in the Eck were approximately doubled after feeding.

## DISCUSSION

The findings described above indicate that Eck fistula formation and the resultant decreased circulation of blood through the liver does not in itself elevate the fasting free amino acid levels in plasma of dogs fed a diet consisting of bread and meat.

TABLE 5. EFFECT OF A 50 GM/KG. RAW HORSEMEAT MEAL ON PLASMA FREE AMINO ACIDS IN DOGS

AMINO ACID μg/ml. plasma	DOG 51 BEFORE	(ECK FISTULA) AFTER <sup>2</sup>	DOG 61 BEFORE	(INTOX. ECK) AFTER	DOG 63 BEFORE	(NORMAL) AFTER
Threonine.....	14.7	34.9	14.8	27.5	15.1	28.0
Tryptophane.....	14.1	19.4	19.7	37.5 <sup>1</sup>	11.1	37.5 <sup>1</sup>
Methionine.....	14.7	22.9 <sup>1</sup>	13.8	27.6 <sup>1</sup>	8.2	16.8
Tyrosine.....	28.0	43.7 <sup>1</sup>	38.7	70.9 <sup>1</sup>	10.5	16.6
Proline.....	15.7	38.3 <sup>1</sup>	25.8	38.4 <sup>1</sup>	23.2	31.0
Histidine.....	28.0	69.0 <sup>1</sup>	23.3	64.5 <sup>1</sup>	18.5	28.4
Arginine.....	21.6	42.6 <sup>1</sup>	48.0	63.0 <sup>1</sup>	42.6	47.4
Phenylalanine.....	16.8	34.1 <sup>1</sup>	9.9	12.6	11.2	11.4
Valine.....	16.2	17.7	24.6	41.5 <sup>1</sup>	33.7	45.0 <sup>1</sup>
Leucine.....			38.7	41.4		
Isoleucine.....			12.2	18.0		

<sup>1</sup> Above upper limits of fasting range.    <sup>2</sup> Blood samples were taken 5 hours following the meal.

This is in agreement with the previous work of Whipple and Van Slyke on blood amino nitrogen. Meat feeding, however, was associated with a demonstrable difference between Eck fistula and normal animals. The former exhibited increased fasting levels of several amino acids during meat intoxication. Blood levels 5 hours after feeding also were shown to be higher in the dogs with Eck fistula. The 5-hour period was selected in view of the report of Van Slyke and Meyer (6) that blood amino nitrogen was approximately doubled at this time following a meal of raw beef.

Dogs with obstructions of the portal vein ordinarily have a greater tolerance for the raw meat diet than has the Eck fistula animal. Dog 50 of our series is an exception in that this animal developed unmistakable signs of meat intoxication. The histidine content of the plasma was elevated, as in the majority of other animals with meat intoxication.

The rise in plasma free amino acids in meat-intoxicated dogs is probably an indication of the decreased deamination which results from loss of the portal blood supply to the liver. The alterations in dye clearance and serum phosphatase (7) are further evidence of impaired liver function in the Eck fistula animal. Whether or not

elevated levels of specific amino acids are in themselves responsible for the intoxicated condition of the animals might be determined by feeding or injection studies with the compounds which were shown to increase significantly.

#### SUMMARY

Data have been compiled on fasting levels of 16 free amino acids in the plasma of normal dogs. Five of 6 amino acids studied at intervals in the same animals were shown to be relatively constant. Dogs with simple portal obstructions, fed a diet of raw horsemeat, showed variable alterations in amino acid levels. One of 6 dogs in this series became intoxicated but recovered. Normal dogs on the same diet showed decreases in several amino acids. The formation of an Eck fistula did not result in elevated plasma free amino acids. Eleven of 13 dogs with Eck fistulas showed signs of meat intoxication on a diet of raw horsemeat. During intoxication they exhibited increased plasma free histidine, tyrosine, methionine, leucine, glycine, arginine, cystine, phenylalanine, serine and proline. The 2 dogs which did not intoxicate showed an elevated plasma histidine and a decreased threonine. Other amino acids were relatively constant, even after 20 days on the diet. Both normal and Eck fistula animals showed a definite rise in the plasma free amino acids after a meal of raw horsemeat. The rise in levels of 6 amino acids was greater in Eck fistula dogs than in the normal control.

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# BROMSULPHALEIN REMOVAL RATES DURING HYPOTHERMIA IN THE DOG<sup>1, 2</sup>

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IT IS generally agreed that a depression in body metabolism, as indicated by a reduced oxygen consumption, is usually found in animals with a rectal temperature of 25° C. or lower. Above this level a particular function may show a depression or an elevation. Dill and Forbes (1) have shown that the total energy exchange in prolonged hypothermia in humans with various subnormal body temperatures down to 25.5° C. may be two and even three times the basal normal. In dogs undergoing progressive cooling Penrod (2) has found an increased oxygen consumption that reaches a peak at about 28° C. rectal temperature. It is concluded in both of these experiments that the oxygen consumption is roughly proportional to the degree of shivering. Rosenhain and Penrod (3) report a progressive fall in cardiac output of dogs after 35° C. At this temperature and above, it may be increased, although the pulse rate begins to fall approximately linearly soon after the onset of cooling.

It was thought that a study of the body's ability to remove a foreign substance such as bromsulphalein might yield some information concerning the activity of the liver and the other removal sites of the dye during early hypothermia.

## METHOD

In normal mongrel dogs moderately anesthetized with sodium pentothal, bromsulphalein (BSP)<sup>3</sup> removal rate was measured using a single intravenous dose of 5 mg/kg. One to 3 days later the same dogs were again anesthetized and immersed in an iced bath (2-5° C.). The animals were divided into *groups A* and *B*. BSP removal was again measured, the injection being made when the rectal temperature reached 35° C. in *group A* and 30° C. in *group B*. Since these measurements were being made coincident with others, it was not feasible to keep the rectal temperature at a constant level. The temperatures continued to fall at a rate of approximately 0.17° C. per minute during the test.

Another series of 5 normothermic dogs was given 2 BSP tests under sodium pentothal at an interval of 24 hours in order to be sure that the brief interval between

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<sup>2</sup> This material was presented in part at the 1949 meeting of the Federation of American Societies for Experimental Biology, Detroit, and appeared in abstract form in *Federation Proc.* 8: 17, 1949.

<sup>3</sup> The bromsulphalein was kindly supplied by Hynson, Westcott and Dunning, Inc.

tests and the anesthesia had no influence on the marked changes observed during hypothermia.

In the hypothermic dogs the dye injection was given via a carotid artery in order to insure adequate mixing with the circulating plasma. Blood samples for BSP concentration analysis were obtained from an intravenous catheter placed in the right side of the heart where there was a more active flow as well as more thorough mixing of venous blood than in the extremities of the cooled animals.

	6 Minutes	12 Minutes	20 Minutes	30 Minutes
Normothermia Mg./100cc. BSP.	$1.8 \pm 0.1$	$0.6 \pm 0.2$	$0.4 \pm 0.2$	$0.3 \pm 0.1$
35°C. Group A. Mg./100cc. BSP.	$4.5 \pm 1.4$	$2.9 \pm 1.6$	$2.0 \pm 1.2$	$1.7 \pm 1.0$
30°C. Group B. Mg./100cc. BSP.	$4.8 \pm 1.2$	$3.2 \pm 0.9$	$2.5 \pm 1.4$	$2.1 \pm 0.6$

#### BROMSULPHALEIN REMOVAL IN THE HYPOTHERMIC DOG

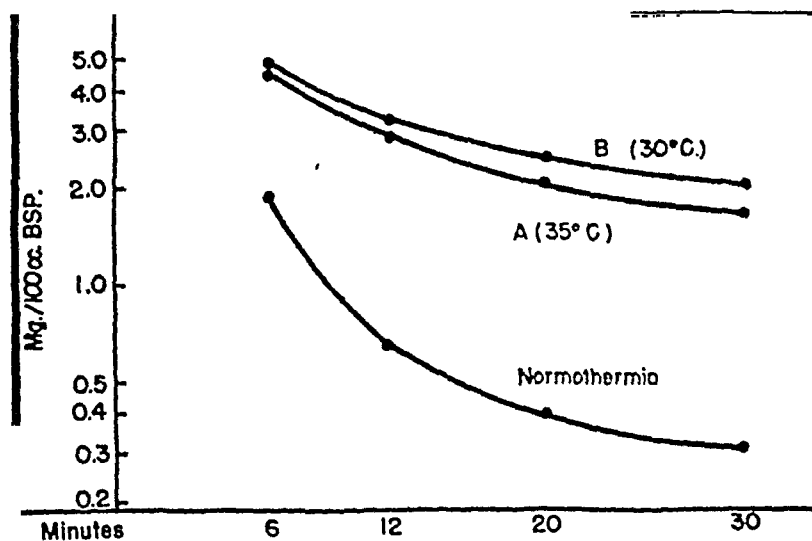


Fig. 1. AVERAGE BSP REMOVAL RATES of 16 anesthetized normothermic dogs, 7 dogs at 35° C (A), and 10 dogs at 30° C. (B). Ordinate: plasma BSP concentration plotted logarithmically. Abscissa time plotted linearly.

Blood samples were obtained at 6, 12, 20, and 30 minutes after the BSP injection in all animals. Plasma BSP determinations were made with a Coleman Jr. spectrophotometer<sup>4</sup> using the technique described by Bradley *et al.* (4). All rectal temperatures were recorded by means of a Leeds and Northrup 'Speedomax' recorder.

#### RESULTS

Figure 1 summarizes the observed data. In the anesthetized normothermic control tests the removal was found to be prompt and rapid, not differing appreciably

<sup>4</sup> This method is accurate to 0.1 mg/100 cc. Most errors will be on the low side of the true concentration. Hemolyzed or turbid samples will falsely show even lower BSP concentrations and hence are not used.

from that of conscious animals and in agreement with the results of other investigators. (BSP concentration 30 minutes after injection was  $0.2 \pm 0.1$  mg/100 cc. and  $0.3 \pm 0.1$  mg/100 cc. in 13 conscious and 16 anesthetized dogs respectively.) As can be seen from the accompanying graph, however, the percentage disappearance rate was not found to be constant as was the case in most of the humans examined by Ingelfinger *et al.* (5). The most rapid disappearance occurred during the first 12 minutes after injection followed by a more gradual decline in dye concentration so that by 30 minutes only a small fraction of the original amount of BSP was present.

The retarded removal rates in the two groups of hypothermic animals roughly paralleled each other with the colder dogs showing a greater retention and a slower rate. In a few animals blood samples were obtained one hour after the dye injection, and during the last 30 minutes only a very small additional amount of BSP was removed.

#### DISCUSSION

The exact mechanism for the BSP retention in hypothermia is not known. Two possible explanations are: 1) Depression of the metabolic activity of the removal sites, and 2) reduction in hepatic blood flow. Fuhrman and Field (6) have shown that there is a marked depression of the metabolism of rat kidney cortex slices and brain tissue as measured by their oxygen consumption and anaerobic glycolysis. The temperatures used by them ( $0.2^{\circ}$  C.) however, were far more severe than those of these experiments.

The presence of shivering with increased oxygen consumption demonstrates that the metabolism of certain body cells is frequently elevated during early hypothermia with a peak oxygen consumption at about  $28^{\circ}$  C. rectal temperature. The glycogen stores of the liver are materially depleted indicating some increase in at least one of the functions of this organ (7).

Since the liver is believed to be the chief site for the removal of BSP, a reduced hepatic blood flow could be largely responsible for the slower removal rates. Mendeloff *et al.* (8) have found a decrease in BSP removal rates in normal humans during exercise. An immediate decrease in BSP removal has been found by Culbertson *et al.* (9) when the liver blood flow is diminished by the vascular adjustments that occur when a patient is tilted from a recumbent position to an upright one. These studies indicate a close relationship between BSP removal and total hepatic blood flow. However, cardiac output studies (3) on hypothermic dogs do not indicate that the total blood flow is materially reduced in the  $35^{\circ}$  C. range, but by  $30^{\circ}$  C. it is reduced by some 23 per cent, a figure in line with the reduced BSP clearance found. At this time the relative importance of diminished cellular activity and decreased blood flow in BSP retention cannot be decided. If the liver shares in the general reduction in cardiac output found after  $35^{\circ}$  C., it would appear that this factor is the more important one.

#### SUMMARY

Plasma BSP removal rates were measured on 7 dogs at  $35^{\circ}$  C. and 10 dogs at  $30^{\circ}$  C. rectal temperature. Both series of animals showed a marked retention and a slowed removal rate. The disappearance rate curves roughly paralleled each other with the  $30^{\circ}$  C. group showing a greater retention and a slower removal rate. De-

creased cellular activity or reduced hepatic blood flow or both may be responsible for the retarded removal rate.

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# INFLUENCE OF GRADED ARTERIAL PRESSURE DECREMENT ON RENAL CLEARANCE OF CREATININE, P-AMINOHIPPURATE AND SODIUM<sup>1</sup>

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IN A previous study designed to establish the relationship of renal blood flow to arterial perfusion pressure in the intact kidney of the dog, it was demonstrated that due to apparent renal autonomy, blood flow was maintained despite considerable decrease in perfusion pressure (1). This resulted in a curvilinear pressure-flow relationship convex toward the pressure axis. Since direct blood flow measurements only were made in this study, no conclusion could be drawn with regard to the behavior of the renal arterioles in this mechanism. Forster and Maes (2) studied the effects of elevation of mean arterial blood pressure on the clearance of creatinine and p-aminohippurate (PAH) in rabbits whose kidneys had been denervated and whose adrenal glands had been demedullated. They found that when blood pressure was elevated by neurogenic mechanisms resulting from clamping of the carotid arteries that these clearances remained remarkably constant. This apparent constancy of glomerular filtration rate and effective plasma flow appeared to result from increase in afferent arteriolar resistance.

In the present study, the effect of graded decrease in arterial blood pressure on the clearance of PAH, taken to measure effective plasma flow, and on creatinine clearance, measuring glomerular filtration rate, was studied in dogs. In addition, the reduction in glomerular filtration which accompanied the decrease in arterial pressure afforded an opportunity to examine the effect of reduced sodium load on the renal mechanism for sodium excretion. This was of particular interest because of the phenomenon of sodium retention accompanying the reduction in glomerular filtration rate and effective plasma flow noted in congestive heart failure (3, 4).

## METHODS

Female dogs averaging 16.5 kg. in weight (range, 11.5 to 23.5) were used. They were anesthetized with 30 mg/kg. of pentobarbital sodium administered intravenously. The left kidney and dorsal aorta were exposed by a dorsal retroperitoneal approach. The left ureter was catheterized so that the tip of the catheter lay within the renal pelvis; the length of the catheter was kept to a minimum so that the dead space of the collecting system was kept to a negligible volume. With the exception of several of the earlier experiments, the right kidney served as a control. Its urine was collected by means of an indwelling bladder catheter.

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Arterial pressure decrement to the left kidney was produced by a tourniquet around the dorsal aorta just between the right and left renal arteries, made possible by the higher origin of the right renal artery. Gradual occlusion of the aorta by the tourniquet thus decreased arterial inflow pressure to the left kidney but kept the blood supply to the right kidney reasonably constant. Carotid mean blood pressure was taken as the index of arterial pressure to the right (control) kidney, and the femoral mean blood pressure was taken as the index of arterial pressure to the left (experimental) kidney. The validity of the latter procedure was tested in 3 dogs by introduction of a long cannula into the abdominal aorta via the femoral artery, ligated in position just behind the axis of the left renal artery. The results of these experiments were in accord with those in which renal arterial inflow pressure was measured via the femoral artery.

The plan of each experiment was to follow a pair of control periods with four stages of graded arterial pressure decrement, with two consecutive urine collection periods at each level, followed by return of arterial pressure to control levels with two final recovery urine periods. Adequate discard periods were observed between each level of arterial pressure, with longer periods during stages of low urine flow. To insure adequate urine volumes, all animals were hydrated with 200 to 300 cc. of 0.9 per cent saline prior to the initial urine period, and a moderate amount of mannitol was included in the infusion fluid containing creatinine and PAH so that about 12 mg/min/kg. of body weight were given following a 5-gm. priming dose. Constant infusion was obtained by use of a mercury pump. Control urine flow averaged 2.0 cc/min. for the left kidney. Bloods were taken before and after each pair of urine periods, and interpolated values corrected for emptying time were used for calculation of the clearances.

The method of Smith *et al.* (5) was used for PAH analysis. Plasma PAH determinations were done on  $\text{CdSO}_4$  filtrates. Creatinine was measured by the alkaline picrate method (6). Sodium tungstate filtrates were used for plasma creatinine determinations. All analyses were made in duplicate. Sodium was analyzed with a Perkin-Elmer model 18-A flame photometer<sup>2</sup> on diluted urines and plasmas; in some cases, trichloroacetic acid filtrates were used for plasma sodium analysis. No difference from direct plasma analysis was noted. When sodium clearances were calculated, a plasma sodium correction for the Donnan effect was made by the factor:  $KP/W$ , in which  $K_{\text{Na}} = 0.925$ , and  $W$  (percentage of water in the plasma) was taken as 0.94.

## RESULTS

*Effect of Graded Arterial Pressure Decrement on Clearance of PAH and Creatinine.* Ten animals are included in this series. In three earlier experiments control clearances on the right kidney were not made simultaneously with the experimental changes produced in the left kidney by decreased arterial pressure, hence systemic factors which might alter clearances could not be detected. Although these three experiments are in approximate agreement with those done later, the emphasis of this section will

<sup>2</sup> We are indebted to Dr. Viola Startzman, Dept. of Pediatrics, for use of the Perkin-Elmer flame photometer.

be placed on seven experiments in which both right and left kidney clearances were followed simultaneously.

A representative experiment is illustrated in figure 1. This experiment is particularly instructive because systemic factors which might influence renal blood flow appear to be absent during the 3-hour duration of the experiment, as evidenced by the constancy of the control clearances. Thus it can be concluded that alterations

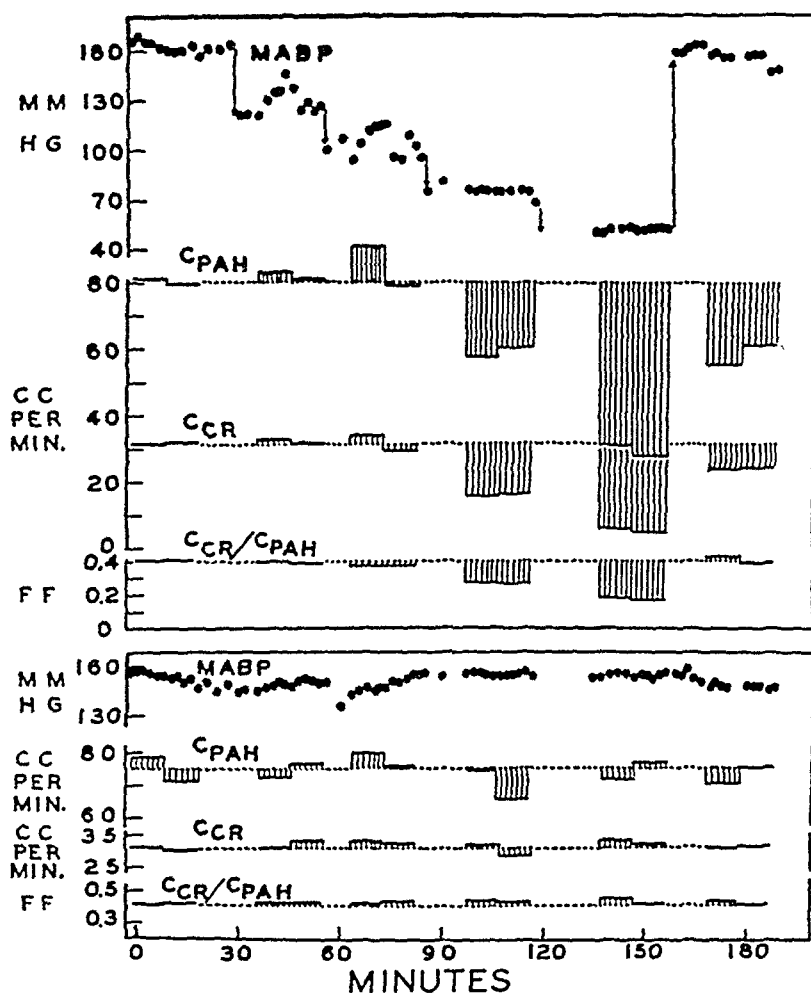


Fig. 1. EFFECT OF GRADED ARTERIAL PRESSURE decrement on renal clearance of PAH and creatinine, and on filtration fraction. *Upper*: experimental (left) kidney. *Lower*: control (right) kidney. Dashed lines in all cases designate the control data averages.

noted in the experimental kidney clearances must be specifically due to intrarenal changes resulting from the influence of decreased arterial pressure. The control clearances of PAH and creatinine, together with the filtration fraction, appear in the lower part of the figure in relation to the control arterial pressure.

In the upper part of figure 1 it is seen that as arterial pressure is progressively decreased from a mean of 162 mm. Hg through stages averaging 127 mm. and 104 mm. respectively, there are no significant changes in PAH and creatinine clearance, and the filtration fraction remains constant. At the level of 73 mm. Hg, however, clearances begin to decrease noticeably, and are significantly lower at a level averaging

50 mm. Hg. During the latter stages, the clearance of creatinine falls somewhat more rapidly than that of PAH, with the result that the filtration fraction decreases, a finding typical of all experiments. With release of the tourniquet, clearances recover to 78 per cent of the control kidney values. (In all experiments, average recovery for PAH clearance was to 89 per cent of the control kidney, and to 81 per cent of control for creatinine clearance.)

Systemic factors were frequently found to be operative which tended to decrease clearances somewhat during long experiments. In order to correct for this trend the experimental (left) kidney clearances are presented as a ratio to the control (right) kidney in figure 2 for all experiments. Each symbol in the figure represents the average of two consecutive urine collection periods. It is evident that the PAH clearance is well maintained as pressure is decreased to about 100 mm. Hg, then decreases rapidly with further decreases in pressure. Creatinine clearance is maintained to about 120 mm. Hg, then decreases somewhat more rapidly than the PAH clearance, resulting in decrease in the filtration fraction. At about 60 mm. Hg, all clearances rapidly approach zero.

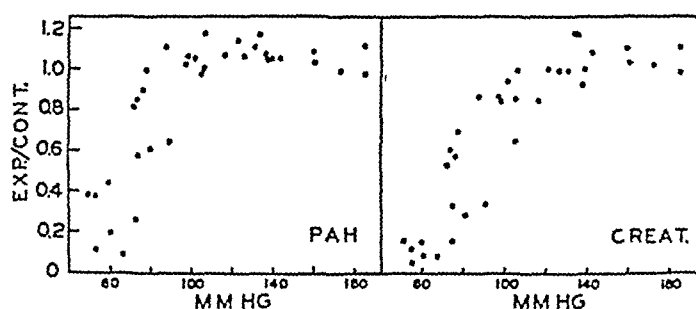


Fig. 2. EFFECT OF DECREASED ARTERIAL PRESSURE ON PAH and creatinine clearance expressed as a ratio to the control kidney. Each point is the average of 2 consecutive urine periods. Data are from 7 experiments.

*Changes in Regional Renal Vascular Resistance Resulting From Graded Arterial Pressure Decrease.* Changes in afferent arteriolar resistance ( $R_A$ ), efferent arteriolar resistance ( $R_E$ ), post-arteriolar resistance ( $R_V$ ), and total renal resistance were analyzed by means of Lampert's equations (7).<sup>3</sup> The same experiment graphically presented in figure 1 is used to exemplify the trend of resistance changes in figure 3. In the upper half of the figure PAH and creatinine clearance are given in cc/min/gm.

<sup>3</sup> Calculation of regional renal vascular resistance in mm. Hg/cc/min. was made according to the following original equations of Lampert (7), with minor modifications cited below:

$R_A = (P_M - P'_0 - 23 \text{ Hc} - 20)/\text{HD}$ ;  $R_E = (1 - 0.47F) (P'_0 - P_0 - 23 \text{ Hc} + 10)/\text{HD}$ ;  $R_V = (P_0 - P_V + 20)/\text{HD}$ . Total Renal Resistance =  $R_A + R_E + R_V$ .

$P_M$  = mean arterial blood pressure;  $P_0$  = osmotic pressure of plasma protein, with protein concentration of plasma taken as 6 gm/100 cc.;  $P'_0$  = osmotic pressure of plasma protein after glomerular filtration;  $P_V$  = renal vein pressure; Hc = hematocrit; HD = effective blood flow; F = filtration fraction.

The minor modifications employed in our calculations were to circumvent the spurious negative resistance values observed by Lampert (7) at substantially decreased arterial pressures. One modification substituted a yield pressure of 14 mm. Hg (1) for the value of 20 mm. Hg employed by Lampert, with an adjustment for nonlinearity for values below 80 mm. Hg arterial pressure. A second minor modification was to make intracapsular pressure proportional to glomerular filtration rate, instead of utilizing a constant value of 10 mm. Hg.

of kidney as related to arterial pressure. Here each point is the average of two consecutive clearance periods, and the trend of results is indicated by curves of best

Fig. 3. CHANGES IN REGIONAL RENAL VASCULAR RESISTANCE resulting from graded decrease in arterial pressure. *Upper:* changes in PAH and creatinine clearance in cc/min/gram for the same experiment shown in fig. 1. Each point is the average of 2 consecutive urine periods. *Lower:*  $R_{total}$  is total renal vascular resistance in mm. Hg/cc/min.  $R_A$ , afferent arteriolar resistance;  $R_V$ , postarteriolar resistance;  $R_E$ , efferent arteriolar resistance.

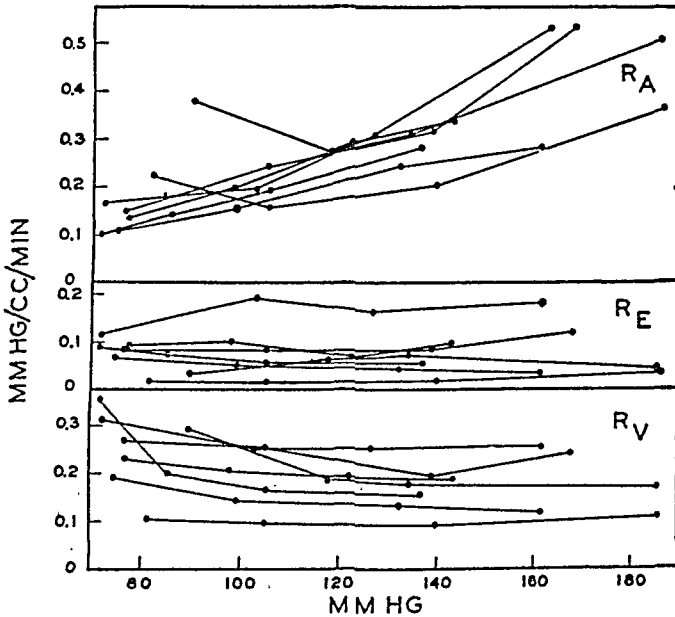
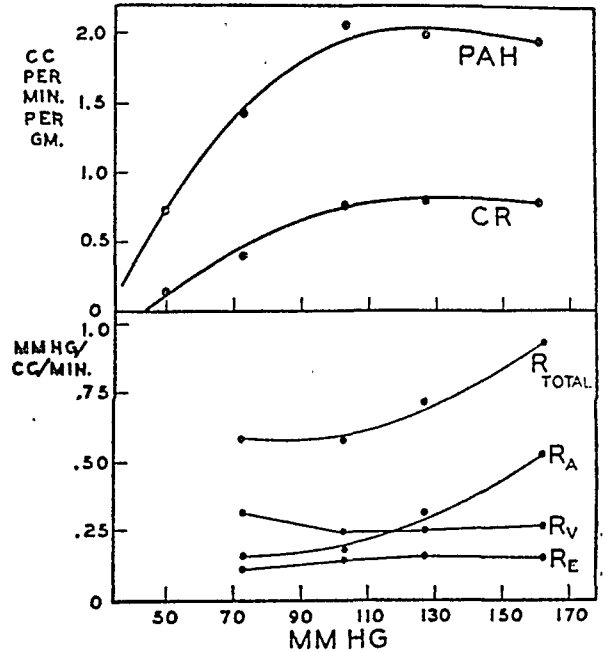


Fig. 4. RESISTANCE CHANGES for experiments shown in figure 2. Each point is the average of 2 consecutive urine periods. (Because of inconsistencies resulting from the small clearance values, calculations for the lowest pressure range are not included.)

fit for the experimental points. The maintenance of clearance during early stages of pressure decrement is obvious.

In the lower half of the figure appear the trends of resistance changes in the kidney. Total renal vascular resistance shows a gradual decrease through the range 162 to 104 mm. Hg, then remains constant to 73 mm. Hg. The decrease in total

resistance appears to be entirely attributable to decrease in afferent arteriolar resistance ( $R_A$ ).  $R_E$  and  $R_V$  show no significant changes, although  $R_V$  may increase a bit at lower pressures. (The data for the lowest clearance figures are omitted in the resistance calculations because small absolute errors in the clearance data give large percentile errors in calculation.)

The conclusion that the constancy of plasma flow and glomerular filtration rate in the earlier stages of arterial pressure decrement is due to decrease in afferent

TABLE 1. REPRESENTATIVE EXPERIMENT SHOWING EFFECT OF DECREASE IN GLOMERULAR FILTRATION ON RENAL CLEARANCE OF SODIUM

(All data for one kidney only: weight 43 gm. Female dog, 13 kgm. body weight.)

PERIOD	MABP	FILT. RATE	SODIUM						
			Plasma conc. <sup>1</sup>	Load	Urine conc.	Urine vol.	Excreted	Reabsorbed	UV/P
	mm. Hg	cc/min.	mM/l.	mM/min.	mM/l.	cc/min.	mM/min.	mM/min.	cc/min.
1	141	39.4	134	5.27	45.2	2.25	0.102	5.17	0.76
2	146	45.3	136	6.13	64.0	2.00	0.128	6.00	0.94
Av.				5.70			0.115	5.58	0.85
3	124	35.9	140	5.05	113.0	1.10	0.124	4.93	0.89
4	121	32.2	142	4.62	77.4	0.95	0.074	4.54	0.52
Av.				4.83			0.100	4.73	0.70
5	99	23.3	138	3.23	20.9	0.50	0.010	3.22	0.0755
6	98	33.3	133	4.58	19.6	0.85	0.017	4.56	0.124
Av.				3.90			0.014	3.89	0.100
7	81	30.5	133	4.05	3.04	0.80	0.002	4.05	0.017
8	74	21.8	136	2.96	4.34	0.60	0.003	2.96	0.018
Av.				3.50			0.0025	3.50	0.017
9	66	8.3	141	1.17	0.00	0.30	0.000	1.17	0.000
10	55	4.0	142	0.58	0.00	0.20	0.000	0.58	0.000
Av.				0.88			0.000	0.88	0.000
11	145	33.0	142	4.70	23.0	1.30	0.030	4.67	0.20
12	138	35.7	142	5.07	39.5	1.40	0.057	5.01	0.40
Av.				4.89			0.043	4.85	0.30

<sup>1</sup> Plasma Na is corrected for the Donnan effect by the factor  $KP/W$ , in which  $K_{Na} = 0.925$ , and  $W$  (% of water in plasma) is taken as 0.94.

arteriolar resistance is confirmed in the combined data shown in figure 4. Note here again the general downward trend of  $R_A$  and the relative constancy of  $R_E$  and  $R_V$ .

*Effect of Reduced Glomerular Filtration Rate on Renal Clearance of Sodium.* In 5 animals gradual reduction in glomerular filtration rate by aortic occlusion was employed to study the effects of reduced sodium load to the renal tubules. Table 1 illustrates a typical experiment. During the control periods the average sodium load is 5.7 mM/min/kidney. Urinary excretion averages 0.115 mM/min. and the plasma clearance of sodium averages 0.85 cc/min. With reduction of load to 4.83 mM/min. urinary excretion decreases to 0.10 mM/min. and clearance to 0.70 cc/min. During the next stage in reduction of glomerular filtration rate, load is diminished

to 3.90 mm/min., urinary excretion is markedly reduced to 0.014 mm/min. and clearance decreases to 0.10 cc/min. as tubular reabsorption of filtered sodium becomes almost complete. This trend is continued in the remaining stages of reduced filtration rate. With restoration of arterial blood pressure and a return of filtration rate toward control values, urinary sodium excretion returns to 0.057 mm/min. and clearance 0.40 cc/min. during the last period when load is 5.07 mm/min.

The relationship of glomerular filtration rate to sodium excretion for all experiments is summarized in figure 5. This shows that sodium excretion diminishes as glomerular filtration rate is decreased from the control average of 40 cc/min./kidney, and that in a range 20 to 30 cc/min. urinary excretion is almost entirely abolished as tubular reabsorption of sodium becomes almost complete at reduced loads.

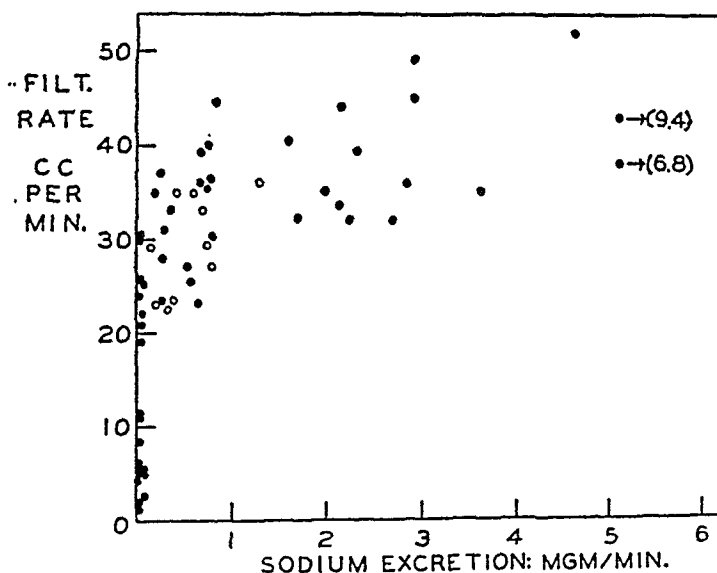


Fig. 5. RELATIONSHIP OF SODIUM EXCRETION to glomerular filtration rate (5 experiments). Open circles: values obtained after return of arterial pressure to normal after experimental changes. It is observed that these data are in the range of normal. Data are for one kidney only.

The relationship of sodium load to urinary excretion and tubular reabsorption is summarized for all experiments in table 2. Here it is revealed that the most pronounced decrease in excretion occurs at the second level of reduced glomerular filtration rate (average, 32 cc/min.), where average excretion is only 0.0173 mm/min. at a load of 4.3 mm/min. At this stage, reabsorption of filtered sodium is 99.5 per cent complete, as compared with 97.3 per cent during the control periods.

#### DISCUSSION

In connection with the previous work (1) employing direct blood flow measurement in analysis of pressure-flow relationship of the kidney, speculation was raised concerning the possibility that changes in blood viscosity resulting from changes in glomerular filtration might be the basis for the apparent renal autonomy of flow. The hypothesis put forward suggested that as arterial pressure was increased, increased filtration of fluid from the plasma at the glomeruli would increase protein and cell concentration of the blood passing through the glomeruli, increasing its

viscosity and thus buffering effects of increased pressure. With reduction in arterial pressure, the reverse effect might be expected to occur. This hypothesis necessarily assumed a linear relationship of glomerular filtration rate to arterial pressure, increases in filtration rate paralleling increase in arterial pressure, and vice versa.

The present investigation has revealed that the above hypothesis is not tenable. Apparently, autonomous renal arteriolar changes are basic to an adjustment of glomerular filtration rate whereby this is kept constant as arterial pressure is decreased, thus precluding changes in blood viscosity. Analysis of the clearance data

TABLE 2. SUMMARY OF RELATIONSHIP OF SODIUM LOAD TO URINARY EXCRETION AND TUBULAR ABSORPTION

	MAP	FILTR. RATE	SODIUM			
			Load	Excreted	Reabsorbed	% Reabsorbed
	mm. Hg	cc/min.	mM/min.	mM/min.	mM/min.	
<i>Control</i>						
	148 (124-176)	40 (32-52)	5.77 (4.7-7.7)	0.160 (0.03-0.41)	5.61 (4.6-7.5)	97.3
<i>Experimental</i>						
1	123 (103-142)	37 (32-45)	5.38 (4.47-6.60)	0.074 (0.03-0.126)	5.31 (4.34-6.57)	98.7
2	96 (83-109)	32 (23-37)	4.30 (3.22-5.48)	0.0173 (0.009-0.035)	4.27 (3.2-5.5)	99.5
3	74 (68-82)	21 (11-30)	2.94 (2.8-4.0)	0.009 (0.001-0.026)	2.93 (2.8-4.0)	99.7
4	58 (44-68)	5 (1-8)	0.61 (0.14-0.8)	0.0013 (0.0-0.1)	0.61 (0.3-1.18)	99.7
<i>Recovery</i>						
	136 (119-156)	30 (22-36)	4.10 (3.0-5.0)	0.026 (.008-.037)	4.07 (3.0-5.0)	99.3

Data taken from 5 animals, for one kidney only. Kidney wt., range 41-43 gm. Each level represents the average of 10 urine collection periods. Figures in parentheses are the range of variations.

by the method of Lampert reveals that this maintenance of glomerular filtration rate (and effective plasma flow) is by afferent arteriolar dilatation. It is interesting to note that Forster's data on the rabbit (2) suggest that the constancy of renal blood flow in the face of increased arterial pressure is by afferent arteriolar constriction. These findings together identify the role of the afferent arterioles as a buffering mechanism which counteracts the effects of changes in systemic arterial blood pressure. Beyond the conclusion that this regulation is definitely intrarenal, no further information can be supplied at present as to the exact nature of this buffering mechanism.

The findings in connection with the alterations in the renal clearance mechanism of sodium during graded arterial pressure decrement throw some light on the problem of sodium retention in congestive heart failure. In this condition, due to reduction in cardiac output, glomerular filtration rate and effective plasma flow are decreased, the latter more so than the former (3, 4). Decreased filtration rate is associated with decrease in urinary excretion of sodium.

Our findings lead to the conclusion that as glomerular filtration rate is decreased, the load of sodium to the tubular cells is decreased, with the result that tubular reabsorption becomes more complete at lower filtration rates. In fact, the present data suggest that an actual 'threshold' for sodium exists, below which sodium reabsorption is complete.<sup>4</sup> The normal kidney offers a load which is above this 'threshold' with the result that small amounts of sodium are normally excreted in the urine. This threshold has been computed for the present series and tentatively set at a value of 3.9 mM. of sodium per minute per kidney (average weight 42 gm.). The load at which sodium excretion begins is delivered to the tubules at a filtration rate of about 25 cc/min., 63 per cent of the control average. This is in no wise to be interpreted as meaning that a 'Tm' (tubular maximum) for sodium exists here, for in the range of the present data tubular reabsorption continues to increase as load is increased, even though accompanied by increased urinary excretion. Higher sodium loads to the tubular cells would be required than attained in the present series to establish the presence of a Tm such as exists, for example, for glucose.

The implication of these findings to the renal mechanism in congestive heart failure is apparent, notwithstanding. Because of reduction of glomerular filtration rate in congestive failure, a smaller load than normal is offered to the tubular cells whose reabsorptive capacity does not appear to be altered. This load is less than the 'threshold' for sodium, and as a result all filtered sodium is reabsorbed and hence retained in the blood by the kidneys. Sodium retention supplies the osmotically active substance needed for the fluid retention which leads to edema formation.

It is interesting to note that when glomerular filtration rate is improved in patients by the use of xanthine diuretics (4) that sodium excretion increases noticeably. This may be assumed to mean that the increase in sodium load resulting from increased filtration exceeds a threshold such as exists in dogs. Likewise, when glomerular filtration rate in patients normally edema-free is reduced by exercise below a critical level of 70 cc/min., sodium retention and edema formation begin (8), also supporting the concept that a threshold exists below which sodium reabsorption is complete.

#### SUMMARY AND CONCLUSIONS

When arterial infusion pressure to the kidney is gradually reduced by gradual aortic occlusion, clearances of p-aminohippurate and creatinine are well maintained

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<sup>4</sup> 'Threshold' has been defined elsewhere as a critical plasma concentration below which tubular reabsorption of certain filtered constituents is complete, and above which urinary excretion begins. Since plasma sodium concentration remains essentially constant in these experiments, but filtration rate is varied, we employ the term 'threshold' to designate a critical *load* (plasma concentration X filtration rate) below which sodium reabsorption is complete and above which urinary excretion begins.

near control values through a range of about 150 mm. Hg to 100 mm. Hg. Subsequently, clearances decrease as glomerular filtration rate and urine flow cease at about 60 mm. Hg. During this latter phase, creatinine clearances fall more rapidly than the PAH clearances, so that the filtration fraction decreases.

Calculation of renal resistance changes by the method of Lampion indicates that maintenance of renal clearances is due to afferent arteriolar dilatation; efferent arteriolar resistance and post-arteriolar resistance remain essentially constant. This emphasizes the role of the afferent arterioles as a buffering mechanism to maintain blood flow and glomerular filtration in opposition to systemic arterial blood pressure changes.

Renal excretion of sodium decreases as glomerular filtration rate is decreased by aortic occlusion. This is because as filtration is reduced, the sodium load to the tubular reabsorptive mechanism is decreased with the result that tubular reabsorption becomes more complete. The data suggest that a 'threshold' for sodium reabsorption exists at a load of about 3.9 mm/min/kidney below which reabsorption is complete. The normal kidney offers a load somewhat above this threshold so that small amounts of sodium are normally excreted. The significance of these findings as they bear on the problem of renal sodium retention in congestive heart failure is discussed.

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# VALIDITY OF PULSE CONTOUR METHOD FOR CALCULATING CARDIAC OUTPUT OF THE DOG, WITH NOTES ON EFFECT OF VARIOUS ANESTHETICS<sup>1, 2</sup>

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THREE years ago (1) we presented a procedure by which the stroke index of the dog could be estimated from the contour of the aortic pressure pulse. Since, in the dead dog, the contracted and the relaxed aorta showed very similar increments in volume with a given increment in pressure, it was assumed that these volume increments would not be changed in significant degree in the living dog under various physiological conditions. The application of this assumption to the calculation of the stroke index from aortic pressure change proved successful.

To perform this calculation, the arterial bed was divided into four parts, the transmission time to each part being estimated from average pulse wave velocity figures. These respective times were then applied to an aortic pressure pulse to indicate the corresponding pressure increase above diastolic in each part at the end of ejection. The volume uptake of each part was obtained by referring the respective pressure increments to the table of volume change per unit pressure rise for each part, which table was derived from measurements made on dead dogs. To the summated uptakes was added an estimated systolic drainage volume. The stroke indexes, which were the sum of the blood taken up by the distended arteries and the blood drained out through the arterioles during systole, showed surprisingly good agreement with those given by the dye injection technique in 45 cases.

This agreement would imply that even though tone changes might occur in the arterial bed, they did not alter to a significant extent the net volume uptake per unit pressure rise. While, in these 45 cases, we had employed various means to change the stroke index considerably, the experiments were not specifically designed to emphasize conditions where tone change might be assumed to be great. The series has therefore been expanded with that goal in view, and also to test the applicability of the method at high and low blood pressure levels, which had not been adequately determined.

## METHODS

A total of 187 comparisons, using 66 dogs, have been made between stroke indexes estimated from the pressure pulse contours, and those given by more direct

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measures. In 61 cases, the dye injection technique<sup>3</sup> (2) was used. In the other 126 cases, the direct Fick procedure, using oxygen, was employed. Oxygen uptake was recorded from a Sanborn spring-balance spirometer, connected to an intratracheal cannula. Venous blood was collected through a catheter whose tip lay either in the outflow tract of the right ventricle or in the pulmonary artery, as determined by fluoroscopy. In all determinations, a continuous recording of aortic pressure was made, by a sound passed down the left carotid artery into the ascending aorta, connected to an optical manometer.

All dogs were anesthetized during the procedure, the usual routine being the use of 10 mg/kg. morphine and 15 mg/kg. sodium pentobarbital (I.V.). In some cases morphine only was used, and in others ether, or urethane, was employed. In the first 45 cases, already published, various drugs and hemorrhage were used to vary the stroke index. Three general experimental procedures were used for the rest of the comparisons. 1) Dogs were subjected to serial hemorrhages, comparisons being made before hemorrhage and at selected times during the course of the bleeding. In this category are 23 control, and 75 post-hemorrhage determinations. In 36 of the 75 cases, the animals had received 5 mg/kg. Dibenamine<sup>4</sup> 30 minutes before the start of the hemorrhage. 2) Twenty-nine comparisons were made on dogs after both carotid sinuses had been resected, and both vagi sectioned. Sixteen of these were made during the period of acute hypertension, and 13, when the pressure was being reduced from high levels by hemorrhage. 3) Fifteen comparisons were made during the course of a constant infusion of 80  $\mu$ g/min. of epinephrine.

Experience with the method of calculating the stroke index from the pressure pulse, as first advanced, has revealed that it is needlessly elaborate. Uptake of the four arterial beds can be obtained easily and rapidly, while the calculation of the time-pressure areas for the estimation of systolic drainage is cumbersome. In this calculation, outflow is assumed to be proportional to arterial pressure less 20 mm. Hg, and the drainage accomplished after the end of the ejection period taken to be equal to the previous arterial uptake.

Since time is required for the pulse wave to reach the terminal arterioles ( $T_w$ ), the net effective systole ( $T_s'$ ) for the whole of the arterial tree is represented by the first portion of the aortic pulse only, and has a duration of  $T_s - T_w$ , where  $T_s$  is the total length of systole of the aortic pulse. The effective time of diastole ( $T_d'$ ) would then be  $T_d + T_w$ , where  $T_d$  is the length of diastole of the central pulse. Systolic drainage can then be calculated from 
$$\frac{T_s' (P_s' - 20)}{T_d' (P_d' - 20)} \times U$$

where  $P_s'$  and  $P_d'$  are the mean pressure values during effective systole and diastole, and  $U$  is the arterial uptake.

As first described, the time-pressure areas of the numerator and denominator were obtained by dividing the pulse into a series of segments in which pressure change could be regarded as following a straight line. The area of each segment was then calculated on the basis of its being a trapezoid, and the several areas summated.

<sup>3</sup> The Beckman Spectrophotometer used in this study was obtained through a grant from the Division of Grants, National Institute of Health, U. S. Public Health Service, to Dr. V. P. Sydenstricker.

<sup>4</sup> The Dibenamine was generously supplied by Smith, Klein and French Laboratories.

Systolic drainage averages about 16 per cent of the stroke volume. An error of 10 per cent in the estimation of drainage represents but a negligible error in stroke volume. A painstaking calculation of the areas of numerous segments is, therefore, seldom justified. We have modified our procedure to obtain the values of  $Ps'$  and  $Pd'$  as simply as possible, the calculation of time-pressure areas being done only as a terminal step.

In the calculation of the pulse of figure 1, for example, the first step is the recording of  $Pd$ ,  $Ps$ ,  $Ts$  and  $Tc$ , i.e. 106 mm. Hg, 128 mm. Hg, 110 msec. and 480 msec. respectively. The  $Tw$  value for a diastolic pressure of 106 mm. Hg being 58 msec.,  $Ts - Tw$  is 52 msec., which is rounded to 50 msec. The first 50 msec. of the pulse curve is then divided into a convenient number of time-intervals, say 5 of 10 msec. each. The pressure at the diastolic level and at the end of each of these intervals is then recorded, and an average taken. Actually this average is made in terms of

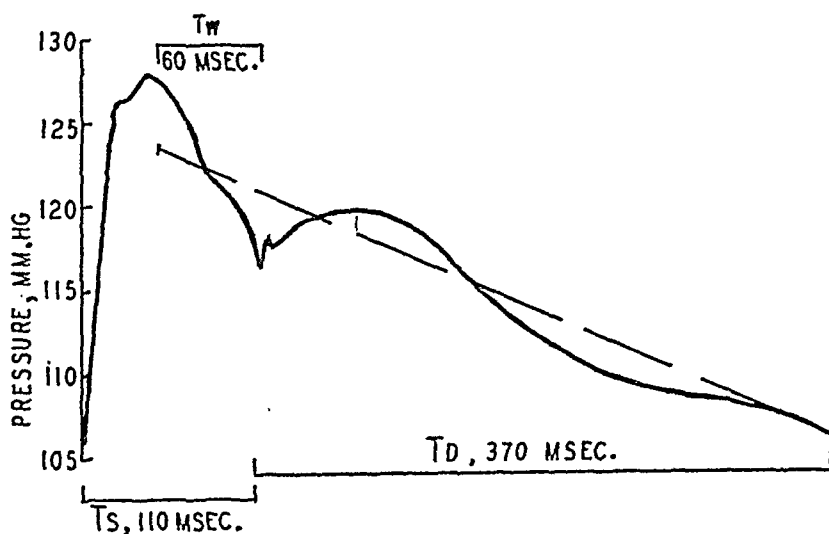


Fig. 1

measuring plate scale units, with a conversion of only the average to pressure units.

For the above pulse,  $Ps'$  would be  $\frac{106 + 116 + 126 + 127 + 128 + 127}{6} = 122$  mm.

Hg. A straight line is then projected by eye from the end of diastole to the start of  $Td + Tw$ , such that the pressure values above and below the line are about evenly matched. The average pressure for this interval is then obtained from the values at

each end of this line, i.e.  $\frac{123 + 106}{2} = 115$  mm. Hg. =  $Pd'$ . These values can now be

fitted into the formula:  $\frac{50 (122 - 20)}{430 (115 - 20)} \times U$  to give the systolic drainage.

#### RESULTS

*Comparison of Contour with Dye Injection or Fick Stroke Indexes.* The deviation of the calculated stroke index from the dye injection or Fick value is expressed in terms of percentage of the letter. In table 1A are shown these percentages as grouped in ascending order of the mean pressure level. In table 1B, the grouping is in ascending order of the stroke index. In neither case is there any consistent trend away from

the reference value. No experimental procedure employed has consistently worsened the fit. We believe, therefore, that the contour calculation will afford a reasonably accurate measure of the stroke index between mean pressure values of 23 and 200 mm. Hg, and that the errors involved are seldom greater than those found when any two measures of cardiac output, e.g. the dye injection and Fick procedures, are compared on the same individual (3). Admittedly, neither the dye injection nor the Fick procedure can test whether stroke indexes calculated from beat to beat are accurate. Both are relatively long-term procedures, the dye injection being more rapid than

TABLE 1. COMPARISON OF STROKE INDEXES OBTAINED BY DYE INJECTION OR FICK PROCEDURES WITH THOSE CALCULATED FROM PRESSURE PULSE CONTOURS

A							B					
NO.	MEAN PRES- SURE	STROKE INDEX, D OR F	STROKE INDEX, CONT.	AV. ERROR	RANGE OF ERROR		NO.	STROKE INDEX, D OR F	STROKE INDEX, CONT.	AV. ERROR	RANGE OF ERROR	
					%						%	
	mm. Hg.	cc.	cc.	± %	-	+		cc.	cc.	± %	-	+
10	23	4.0	3.8	10.8	31	20	10	3.0	3.2	10.0	3	27
10	33	4.6	4.6	10.1	19	27	10	4.0	3.8	13.7	31	18
10	49	8.3	8.1	8.9	11	38	10	5.1	5.5	14.3	30	45
10	57	9.0	8.9	4.8	4	15	10	6.6	6.9	14.9	19	33
10	68	9.0	8.6	9.9	5	22	10	7.7	7.9	13.8	10	41
10	76	13.6	14.0	16.2	13	53	10	8.6	8.8	6.2	1	26
10	83	16.2	16.0	8.6	17	15	10	9.5	9.8	15.9	13	46
10	87	21.5	21.7	9.7	7	21	10	10.6	10.6	10.5	24	28
10	92	24.5	23.6	11.2	20	37	10	12.0	11.8	9.3	24	2
10	96	32.5	32.1	8.0	12	19	10	13.4	13.3	8.1	31	15
10	100	28.8	28.9	9.2	16	20	10	14.8	14.7	10.1	25	14
10	104	26.4	26.5	12.3	23	30	10	16.3	15.9	12.7	35	0
10	108	21.8	21.7	7.7	26	11	10	18.1	18.0	12.9	42	53
10	115	24.3	24.6	10.5	22	24	10	22.6	22.3	11.3	26	14
10	123	23.9	23.9	9.7	50	10	10	26.8	26.4	7.5	50	10
10	131	20.8	22.2	17.5	42	42	10	29.8	30.0	9.8	21	34
10	148	17.7	17.9	13.9	34	33	10	36.3	36.3	8.8	21	13
10	171	16.4	16.8	9.1	21	12	10	43.0	43.3	3.5	7	8
7	211	16.0	15.8	14.3	20	45	7	50.8	51.2	9.1	21	14
Av...		18.1	18.2	10.1				18.1	18.2	10.1		

the Fick. Pulse contours often change during the course of the measurement. While an average of many calculated stroke indexes over the course of the measurement checks with that given by the reference procedure, a single pulse taken at random from the record may be considerably in error.

The calculation has been used by two other laboratories. Huggins, Handley and La Farge (4), in a small series of cases, found agreement with the direct Fick as good as those given here. Duomarco *et al.* (5), using a metered flow into the heart in open-chest dogs, found large discrepancies. We have no evidence to indicate why the technique failed so badly in their hands.

*Normal Cardiac Index Values for the Dog.* Even when the same procedure of

flow measurement is employed, cardiac index values given for dogs by different investigators have varied quite widely. Wiggers (6) has presented a summary table showing average values ranging from 2.63 to 6.41 l. What seems not to have been appreciated is that the anesthetic employed can explain much of this difference. In the table cited, animals under barbiturate anesthesia show low cardiac index values, those under morphine sedation intermediate values, and those under ether high values. Unanesthetized dogs, as perhaps might be anticipated, show values ranging from the highest to the lowest. In table 2 are shown a series of average values obtained on recently anesthetized dogs, before any extensive experimental procedure had been employed. No claim is made as to comparability of anesthesia levels. Ether was used when entrance into the abdominal or thoracic cavity was desired, and the anesthesia was heavy enough for good muscle relaxation. We do not believe that the

TABLE 2. EFFECT OF ANESTHETICS UPON SOME CARDIOVASCULAR FACTORS IN THE DOG

ANESTHESIA	NO. OF DOGS	MORPHINE DOSE	ANESTHETIC DOSE	PULSE RATE PER MIN.		DIASTOLIC PRESSURE		SYSTOLIC PRESSURE		CARDIAC INDEX		RESISTANCE <sup>1</sup>		LEFT VENTRICLE WORK	
				Mean	S.D. <sup>2</sup>	Mean	S.D. <sup>2</sup>	Mean	S.D. <sup>2</sup>	Mean	S.D. <sup>2</sup>	Mean	S.D. <sup>2</sup>	Mean	S.D. <sup>2</sup>
		mg/kg.	mg/kg.			mm. Hg		mm. Hg		L.				gm. m/sec.	
Morphine	10	10		70	29	74	9	140	24	2.63	0.39	1.81	0.36	4.10	0.41
Urethane	8	10	500	94	38	83	22	129	17	2.77	0.68	1.70	0.45	4.10	0.17
Sodium pentobarbital	47	10	15	107	36	80	15	117	17	2.81	0.75	1.81	0.57	5.90	0.29
	5	10	30	177	35	88	15	116	17	2.63	0.60	2.08	0.80	5.20	0.24
	6	0	30	191	31	129	15	150	17	1.82	0.45	4.00	1.03	4.80	0.18
Sodium barbital	7	0	180	156	35	108	16	133	17	2.24	0.60	3.04	0.80	5.00	0.30
Ether	20	10		126	42	88	15	133	21	3.68	1.02	1.46	0.54	6.84	0.17

<sup>1</sup> Calculated as mean pressure—20 mm. Hg/flow/sec./sq. M.

<sup>2</sup> Standard deviation.

differences between anesthetic regimes can be attributed to different depths of anesthesia, however. Increasing the dose of barbiturate, or deepening the ether anesthesia makes even larger the differences in flow, resistance and pressure under the two agents.

The values given in table 2 can be grouped into three general categories. The largest cardiac indexes, and lowest resistance values, are seen with ether anesthesia. In general, the deeper the anesthesia, the larger the cardiac index. In the first stages, the increased flow might be attributed to the cardioacceleration which is present in the excitement phase. Later, the heart becomes slow and the pulse pressure becomes very large. The steadily falling resistance denotes a direct dilator action of the anesthetic.

Intermediary flow and resistance values are seen with morphine sedation, or morphine coupled with urethane or a small dose of sodium pentobarbital. While the averages are not significantly different, there is a tendency for the dogs under mor-

phine alone to have lower flows. This is probably directly related to the very slow heart rates. An acceleration is almost invariably accompanied by an increased cardiac index. Atropinization also generally gives an increased flow.

The larger doses of barbiturates give quite a different picture. The immediate response to an intravenous injection is, as is well known, a decline in pressure. This decline is accompanied by an increased cardiac index. This reaction is but temporary and the pressure stabilizes at a level appreciably higher than those found with the other anesthetics. When the barbiturate is given intraperitoneally, the initial hypotensive period may not be seen. The elevated pressure level is the result of an increased resistance, for cardiac index levels are depressed despite the increased heart rate. It is common knowledge, too, that an animal receiving barbiturate in large amounts will often show a gradually developing circulatory failure. In this decline, the resistance remains high, the pulse pressure becoming progressively smaller. The cardioacceleration has been ascribed to a blocking, by the barbiturate, of the efferent vagus action on the heart (7). The only clue we can offer as to the mechanism underlying the increased resistance is that an epinephrine-blocking dose of 15 mg/kg. Dibenamine will lower the resistance, and increase markedly the cardiac index.

#### SUMMARY

A somewhat modified procedure is given for the calculation of the stroke index of the dog from the contour of the aortic pressure pulse. The quantitative validity of the method has been tested by 187 simultaneous comparisons with the dye injection or the direct Fick techniques. The average error is  $\pm 10.1$  per cent, and there is no evidence of a systematic error in the contour calculation. Experiments designed to produce tone changes in the arterial bed did not produce significant change in the accord of the calculated with measured stroke index. It is concluded that the contour method is reasonably accurate within a mean pressure range of 23 to 300 mm. Hg.

The basal cardiac index and resistance values of the dog are dependent in large degree upon the anesthesia employed. Ether seemingly acts as an effective vasodilator, with a resulting large cardiac index, low resistance and moderately low arterial pressure values. Barbiturates, in full anesthetic doses, tend to produce elevated arterial pressure, low cardiac index and high resistance values.

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# COMPARISON OF CARDIAC OUTPUT BY THE DIRECT FICK AND PRESSURE PULSE CONTOUR METHODS IN THE OPEN-CHEST DOG

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IN A recent paper (1) the conclusion was drawn from the data presented that the pressure pulse method of Hamilton and Remington for calculating cardiac output (2) is not sufficiently accurate either quantitatively or directionally for use in cardiovascular studies. Their method (1) consisted of measuring the input of blood into the right or left ventricle and calculating the output from aortic pressure pulses. While admitting the procedure was drastic and the dogs deteriorated the viewpoint was expressed that the fundamental requirement of the pressure pulse method was not affected, i.e. "it is only necessary that the blood be ejected into an arterial system with normal and unchangeable volume elasticity constants." The conclusion following from their work is that perhaps as a result of the procedures to which the dogs were subjected the distensibility factors have been altered. If this has occurred the basic assumption of Hamilton and Remington's work has been invalidated. An examination of the pressure pulses shown reveals contours that must be classed as unusual and suggests that possibly some changes in the normal cardiovascular relationships have been induced by the procedures used. The contours may be characterized as showing tremendously large pulse pressures, slow heart rates, very long systolic duration (the majority over 200 m.sec.), steep diastolic slopes despite the very low pressure ranges studied and the slopes show little if any inflection as though the rapid flow rates would continue to very low pressure levels. There is also a relatively high calculated peripheral resistance.

There are at least three possible explanations for such pulses: 1) That contrary to the opinion of Hamilton and Remington distensibility can change markedly in the arterial tree and that in these dogs the aortae were relatively inelastic. This factor could in part explain the straight diastolic pressure descents. 2) In these dogs the peripheral bed was wide open. If this is true, flow should slow quite markedly at pressure values of 20 to 40 mm. Hg (3) and cease at about 20. These curves show no evidence of this. With a high peripheral resistance the cessation of flow should occur around 50 mm. Hg. 3) The aorta was losing blood through some other channel (aortic regurgitation). The meter would not measure such regurgitated blood, whereas the contour method would. Regurgitation would increase diastolic and thus systolic drainage and the resulting stroke volumes would be high.

Regarding the last suggestion, in attempts to utilize the pulse contour method in

humans (4), it was found that patients in chronic congestive failure give falsely high stroke volumes. This phenomenon could conceivably be due to an abnormally inelastic aorta or to regurgitation into a dilated ventricle.

A further reason for uncertainty about the method used by Duomarco *et al.* and the condition of the dogs affecting the volume elasticity constants of the arterial tree lies in the excellent agreement found between simultaneous output comparisons using the dye method (2) and the direct Fick (5) in the intact animal. In the original paper (2) a fairly extensive series of stroke volume determinations were done with an agreement between the two methods of  $\pm 8.2$  per cent (average difference). The series of comparisons with the dye has since been extended and in addition a series of comparisons using the Fick method has been done with an agreement of  $\pm 10.1$  per cent (6). Huggins, Handley and La Forge (5) using the Fick principle found agreement of the order of  $\pm 12.2$  per cent (average difference). In a more recent paper (7) dealing with the effects of various drugs on cardiac output in dogs bled to a mean pressure of 50 mm. Hg and held there for 90 minutes, the cardiac output determined from pressure pulses obtained during the hypotensive period, in every case, compared favorably with published data (8, 9) in which the cardiac output was determined by accepted methods.

For the above reasons the problem was reinvestigated using a different method. In this paper, data are presented comparing cardiac output calculated from pressure pulse contours and the direct Fick method in the open-chest dog.

#### METHODS

The dogs were anesthetized with sodium barbital, 300 mg/kg. A Hamilton manometer (10) of adequate frequency was used to obtain the pulse contours. The cannula leading to the manometer was inserted into the left carotid before the chest was opened and records made prior to and immediately after opening the chest. The azygous vein was tied and large cannulae, one in the superior and another in the inferior venae cavae, were inserted. The cannulae were connected to a rotameter through a 'y' tube and the blood was returned to the heart through a cannula inserted into the right auricle. The rotameter data will be presented in a separate paper. Chlorazol Fast Pink, 80 mg/kg. was used at the anticoagulant.

For the direct Fick, oxygen consumption was measured by connecting the dog to a McKesson Recording Metabolism machine. The  $O_2$  circuit consisted of a hose leading from the output valve of the metabolism machine and connecting to a 'y' tube on the other end of which was a balloon sealed in a bottle. The remaining end of the 'y' tube was connected to the tracheal cannula. Oxygen moved from the machine through the balloon, through a water valve and into the trachea. A tube connecting the other end of the tracheal cannula to the return valve of the metabolism machine completed the circuit. Positive and negative pressure was applied to the balloon through a 'y' tube in the sealed bottle by suitable connections with a respirator. Oxygen consumption was determined in 5-minute periods. The  $O_2$  circuit was tested for leaks after the dog was killed by running the machine for a minimum period of 5 minutes with the dead dog in the circuit. The blood samples were taken in oiled syringes simultaneously from the femoral artery and the rubber tube just above the

cannula that returned blood to the right auricle. This sample did not of course contain blood from the coronary sinus. The syringes were placed on ice as soon as the blood samples were taken. Blood oxygens were determined by the method of Roughton and Scholander (11). Simultaneous with the drawing of the blood samples, pulse contours were recorded.

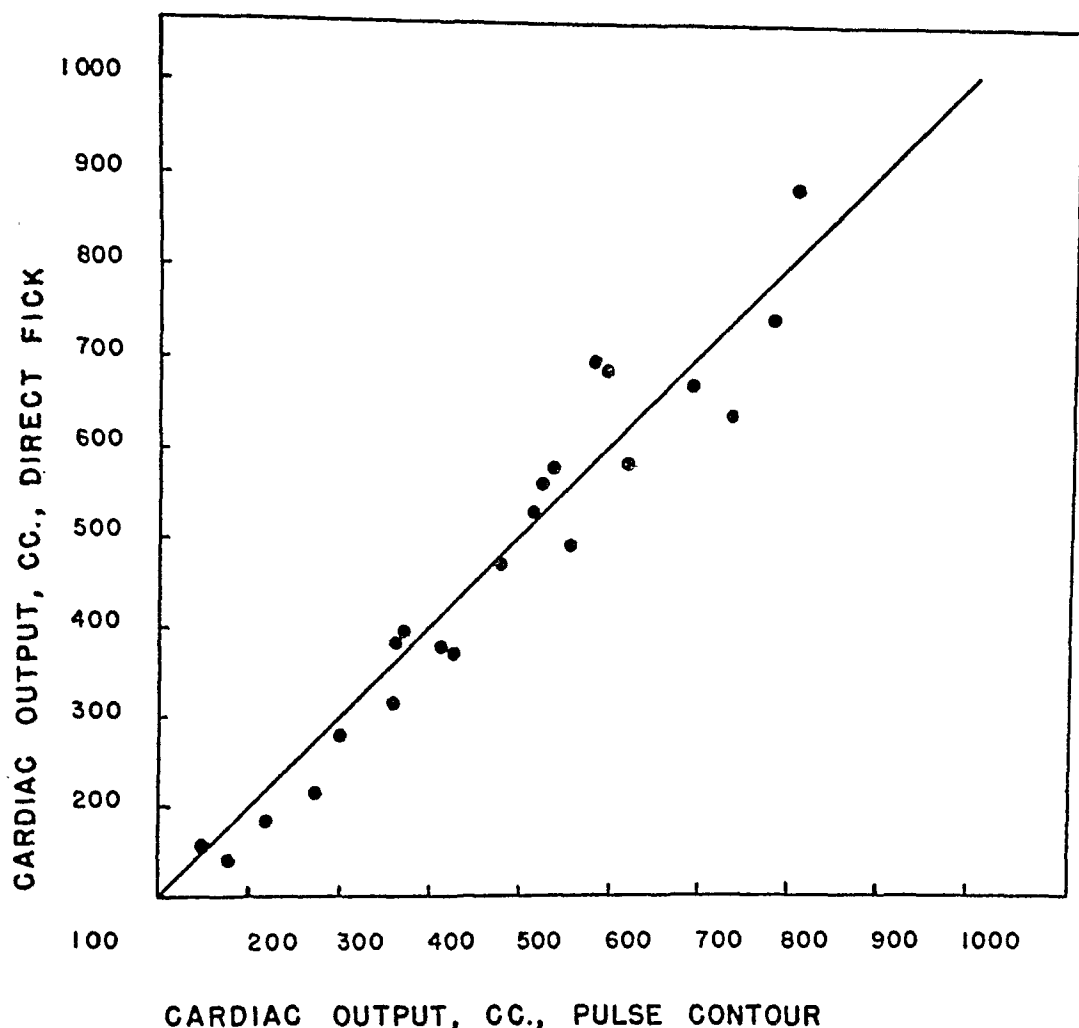


Fig. 1. THE SOLID LINE represents a perfect correlation.

The cardiac output determined by the pressure pulse method is expressed in  $m^2$  of body surface. This was corrected to give a figure for the individual dog to agree with the Fick data.

#### RESULTS

A total of 23 simultaneous cardiac output determinations were made on 5 dogs and the data are incorporated in figure 1. Comparing the direct Fick and the pressure pulse data the average difference is  $\pm 12.5$  per cent with a range from  $-13.7$  to  $+59$  per cent and a coefficient of correlation of  $r = +0.985$ . While the average difference is  $\pm 12.5$  per cent, the pressure pulse method on the average gives values 6.7 per cent higher than the Fick method. This difference can probably be accounted

for by the fact that the pressure pulse method includes the coronary flow while the direct Fick as adapted to this experiment probably does not.

#### DISCUSSION

The data presented here are obviously not in agreement with that of Duomarco *et al.* (1). They found no correlation between the calculated output and measured input while our data indicate an excellent correlation between the calculated output by the pressure pulse method and the direct Fick. The reasons for the differing results is not clear, however, in no instance were pressure pulse contours observed equivalent to the ones published by Duomarco *et al.* Further, the agreement reported here between the measured and calculated cardiac output indicates that the distensibility factors were unaffected by procedures about as drastic as those they used.

In one instance the pressure pulse value is higher than the direct Fick by +59 per cent. If this determination is eliminated from the data the largest deviation is a +27.1 per cent.

#### SUMMARY

The method of Hamilton and Remington for calculating cardiac output from pressure pulse contours agrees closely with the simultaneously determined cardiac output by the direct Fick method in open-chest dogs.

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# COMPARISON OF THE CONSTANT AND INSTANTANEOUS INJECTION TECHNIQUES FOR DETERMINING CARDIAC OUTPUT

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ONE method of measuring cardiac output involves the use of dyes which are known to remain within the cardiovascular tree for prolonged intervals. Measurements are carried out in two ways, either by the injection of dye at a steady rate over a regulated interval (1), or by a single, rapid injection at the beginning of the determination (2). The constant injection method depends upon the establishment of a concentration plateau when the injected substance is quantitatively diluted by once-circulated blood. The existence of such a plateau has been questioned by Hamilton and Remington (3), and these authors doubt the validity of the constant injection technique. The work reported here has been designed to compare the two methods by doing consecutive determinations on the same animal, using the blue dye T-1824.

## MATERIALS AND METHODS

Thirty experiments were carried out on 10 adult mongrel dogs of both sexes, whose weights varied from 11 to 27 kg. Paired experiments were done, an instantaneous injection being followed by a constant injection in each case. Animals were anesthetized initially with intraperitoneal sodium nembutal in doses of approximately 150 mg/kg. and small additional intravenous doses were given as necessary throughout the experimental period. The right external jugular vein was exposed and through a large tributary a no. 8 French ureteral catheter was introduced into the right auricle. A bleeding cannula was inserted into either the right omohyoid or the right common carotid artery.

T-1824 was made into solutions containing either 10 or 15 mg/cc. In the instantaneous injection experiments the dye was injected into the auricular catheter from a syringe calibrated to deliver either 4.60 or 2.65 cc. at the distal end of the catheter. Injection was carried out as rapidly as possible, always being completed in less than two seconds. For constant injections an air pressure device similar to that described by Wiggers (4) was used to give a steady rate of flow. The burette was attached to the auricular catheter through a capillary tube. In these experiments amounts varying from 5.7 to 7.5 cc. of dye were injected over the entire period

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of the determination. The rate was constant for each animal, ranging from 0.3 to 0.4 cc/sec. in separate experiments.

Using a rotating kymograph sampling technique (3), blood samples were collected at measured intervals which were constant in each run and which varied from 0.56 to 0.59 seconds in separate runs. For a cardiac output measurement, 30 such samples of approximately 1 cc. each were collected in tubes, each containing 0.1 mg. of sodium heparin in 0.01 cc. of 0.9 per cent sodium chloride solution. These tubes were centrifuged at 2500 rpm for approximately 15 minutes and a 0.1 cc. aliquot of plasma withdrawn from each. Each aliquot was diluted with 5.0 cc. of 0.9 per cent sodium chloride solution, and its dye concentration determined by measuring its optical density. Measurements were carried out in a Cole-

TABLE 1. COMPARISON OF CONSECUTIVE DETERMINATIONS OF CARDIAC OUTPUT BY CONSTANT AND INSTANTANEOUS INJECTION TECHNIQUES

DOG NO.	WT. IN KG.	CARDIAC OUTPUT		PERCENTAGE VARIATION <sup>1</sup>
		INSTANTANEOUS INJECTION	CONSTANT INJECTION	
		<i>l/min</i>		
1	17.5	3.55	3.45	-3
2	18	3.45	3.15	-9
3	15	3.65	3.35	-8
4	11	2.80	2.50	-11
5	18	2.80	2.80	0
6	16	3.40	3.25	-4
		2.20	3.10	+41
7	27	2.20	2.55	+16
		2.15	2.10	-2
8	18.5	2.35	2.50	+6
		2.25	2.40	+7
9	17.5	3.10	2.65	-15
		2.05	2.25	+10
10	18	2.25	1.90	-16
		2.05	1.95	-5

<sup>1</sup> Measured as percentage variation of constant from instantaneous injection.

man Junior spectrophotometer set at 620  $\lambda$ , and the samples were compared with standard solutions of dye. These standard solutions were prepared by adding known amounts of dye to tubes containing 5.0 cc. of 0.9 per cent sodium chloride solution and 0.1 cc. of the animal's own plasma (5).

All animals were autopsied, and in every case the catheter proved to be in the right auricle.

The cardiac outputs were determined according to the formulae employed by Hamilton and Remington (3).

#### RESULTS AND DISCUSSION

Data on the 30 experiments are shown in table 1.

Figures 1 and 2 show two representative pairs of curves. Constant and instantaneous injection curves are superimposed to show time relationships. Graphs of all

15 constant injection experiments show well defined plateaus similar to those shown in these figures.

It is claimed by Hamilton and Remington (3) that since recirculation occurs so rapidly, a true concentration plateau is impossible. It is clear from the work reported here that such plateaus occur regularly, regardless of the time when recirculation

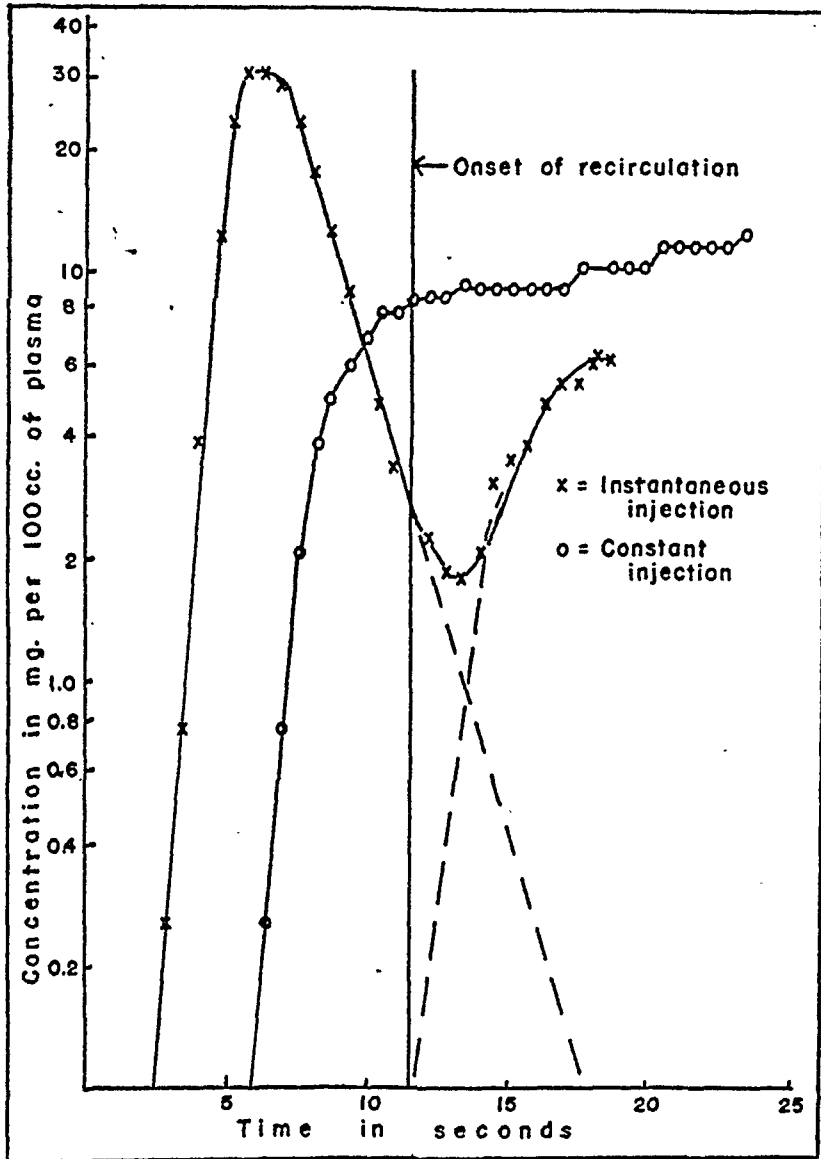


Fig. 1. Dog 2

begins. If the amount of recirculated dye raised significantly the level of the plateau, early recirculation should result consistently in low output determinations by the constant injection method. In this work no such consistently low results have been obtained.

In figure 3 a scatter of the values obtained by the constant injection method as compared with instantaneous injection values is plotted. Examination of the figure and the data in table 1 shows no consistent directional variation between the

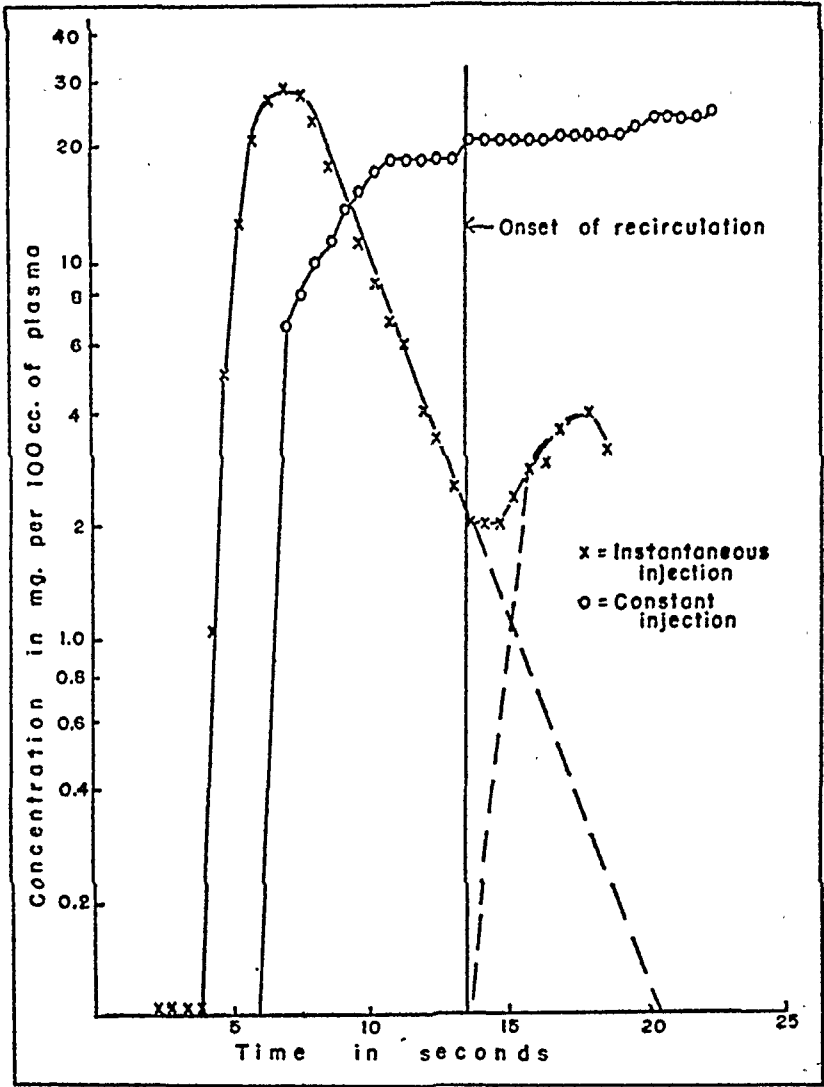


Fig. 2. DOG 10

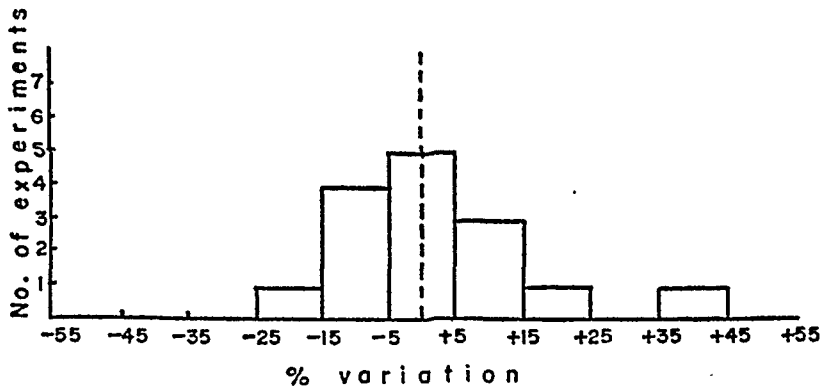


Fig. 3. PERCENTAGE variation of the 15 pairs of experiments

two methods. The variation between the two methods was 10 per cent in this experiment, a figure well within the experimental error of the methods.

## SUMMARY

Consecutive measurements of the cardiac output were carried out by the constant and instantaneous dye injection methods. The results of the two methods agreed within 16 per cent in all but one determination. The distribution of the percentage variation between the two methods showed a normal, symmetrical scatter. The constant injection technique for the determination of cardiac output is as valid as the instantaneous injection method.

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# CHANGES PRODUCED IN HEMATOCRIT VALUE, HEMOGLOBIN AND PLASMA VOLUME BY REPEATED ARTIFICIAL PNEUMOTHORAX

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**A**RTIFICIAL pneumothorax has long been employed as a therapeutic agent. Detailed information concerning physiological alterations induced by pneumothorax is largely limited to the respiratory exchange and pulmonary blood flow. There are a few scattered reports of changes in the formed elements of the blood following pneumothorax. Barker, Ederle and Kircher in 1913 (1) found an increase in the red blood cell count and hemoglobin concentration in dog, rabbit and man as a result of repeated insufflation. Similar results were obtained in the dog by Moog and Pelling (2). Both groups attributed the polycythemic response to anoxia. In man, 3 workers (3-5) described an increased red blood cell count and hemoglobin percentage during repeated pneumothorax, while another (6) found no increase.

The present study represents an attempt to investigate the nature of the reported polycythemia and possible erythropoietic stimulation. Data collected by early workers were confined to measurements of the concentration of formed blood elements and often involved general anesthetics. It was hoped that with the use of the plasma volume determination and other procedures, more information might be obtained.

## METHOD

Closed pneumothorax was induced in 13 dogs, weighing 7 to 12 kg., by injecting room air measured in a 100 cc. syringe and delivered through a 16-gauge needle inserted in the fourth or fifth intercostal space. During this procedure the unanesthetized animals were loosely restrained in the supine position. The needle and syringe were connected with a water manometer. The presence of the unobstructed tip of the needle in the intrathoracic cavity was determined by the smooth respiratory fluctuation of the negative pressure developed in the manometer. In order to maintain a reproducible degree of pneumothorax, the quantity of air delivered at each refill was determined by the elevation of intrathoracic pressure. Injections of 100 cc. to 900 cc. every 2 to 5 days on alternate sides were made to produce an immediate average intrathoracic pressure of +20 mm. H<sub>2</sub>O (expiration to -40 mm. H<sub>2</sub>O (inspiration)). In 2 to 5 days, the corresponding pressures were reduced to approximately -20 mm. and -80 mm. These pressures in the normal dog were -50 mm. and -100 mm. The duration of a course of repeat insufflations varied from 11 to 75 days.

Blood samples were obtained from the jugular vein and femoral artery. Hematocrit percentages were determined in duplicate by the Van Allen tube, oxygen capacity by Grant's method (7), and oxygen content by the Roughton-Scholander technique (8). Plasma volume was measured as described by Gregersen (9) using the blue dye T-1824. The percentage of plasma protein was calculated from the refractive index of plasma determined with Abbe refractometer (10). Reticulocyte percentages were estimated by the method of Osgood (11). An occasional red blood cell count was also made.

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Blood samples were removed at least 48 hours following an insufflation of air in the great majority of cases. This procedure was adopted since the chronic effects were those generally desired.

Pneumothorax was preceded by a control period during which the animals were trained to lie quietly on the board. Blood samples were analyzed every few days for at least three weeks or until the values had stabilized.

### RESULTS

*Conditions of Animals.* An increase in respiratory activity was apparent immediately after insufflation. In 3 animals ventilation was measured quantitatively. It was found that minute volume had increased above that of the control period. The increase was the result of an accelerated respiratory rate and occurred despite a diminished tidal air volume. The percentage oxygen saturation of arterial blood in 18 to 20 measurements made on 5 dogs was within the range of control values. It would appear that the compensatory reactions following the pneumothorax were adequate to prevent anoxic anoxia. Arterial blood pressure measured by direct needle puncture in 4 dogs showed no significant variation from that of the control period.

All animals were maintained in good condition throughout the entire period. Body weight remained rather constant and no untoward effects of the procedure were evident. Since the dogs led a somewhat confined life, no instances of dyspnea on exertion were encountered.

*Immediate Effect on Blood.* Although the primary purpose of this study was to determine the influence of long continued artificial pneumothorax on the formed elements of the blood, some attention was directed toward any immediate effect. A few measurements were made in 5 of the animals at hourly intervals after the primary insufflation and all results were in substantial agreement. Figure 1 gives the results of a primary insufflation of 650 cc. in a 12-kg. dog. Hematocrit percentage, oxygen capacity, red blood cell count and plasma protein percentage were measured several times during a 2-hour control period and for 4 hours after pneumothorax. Another set of measurements were obtained the next day. As shown in figure 1, no significant change occurred in any values as a result of insufflation. In addition, an intravenous injection of T-1824 was made during the control period. The optical density of the dye in plasma yielded the characteristic slow disappearance curve. It will be noted that no shift occurred following insufflation, indicating a lack of marked fluid movement into or out of the vascular bed.

*Chronic Effects on Blood.* Eight series of pneumothoraces, lasting 20 to 75 days, were performed using 7 normal dogs. Hematocrit percentage, oxygen capacity, as well as red blood cell count when occasionally done began to rise 3 to 8 days following the institution of pneumothorax. This rise then persisted from 15 to 30 days after which, if insufflations were continued, all values remained at the elevated plateau. If, on the other hand, insufflations were terminated the blood values returned to those of the control period within 6 to 12 days. The average increase in hematocrit values ranged from +4 to +30 per cent above the control figures with an average increase of 19 per cent. An example of the change in hematocrit percentage, oxygen capacity and red blood cell count following pneumothorax is shown in figure 2.

Of the 8 animals in the series in which pneumothorax was produced, 7 demon-

strated definite increases in the *values* of the various physiologic responses being studied. One animal, *number 6*, showed only +4 per cent increase in hematocrit volume, the next lowest being +12 per cent. It is interesting to note that this animal was the only one in which subcutaneous emphysema was noted following insufflations. Air injected into the intrathoracic cavity of this animal soon leaked into the subcutaneous tissue spaces in large quantities. Intrathoracic pressure rapidly returned to normal and the state of pneumothorax was evanescent (table 1).

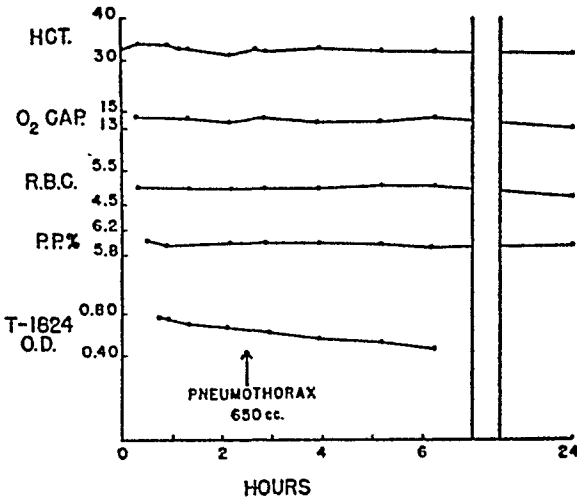
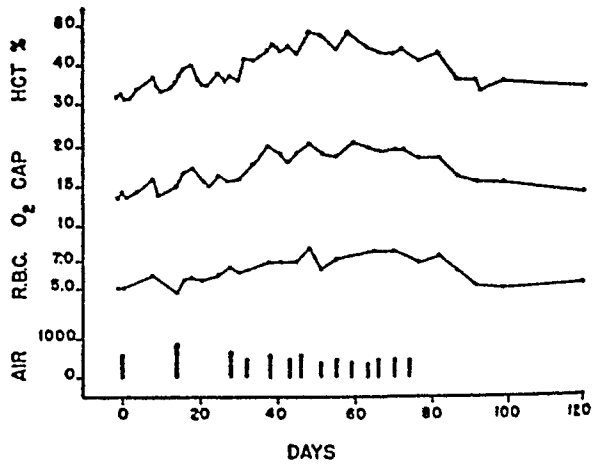


Fig. 1. ACUTE INFLUENCE of a single insufflation on hematocrit percentage, O<sub>2</sub> capacity (vol. per cent), red blood cell count (millions/cu. mm.), plasma protein percentage and optical density of T-1824 in plasma. No significant changes are evident in the concentration of the formed elements and the disappearance rate of T-1824 shows no marked fluctuations.

Fig. 2. EFFECT OF REPEATED AIR INSUFFLATIONS on the formed blood elements (fig. 1). Intervals of 14 days separated the 1st and 2nd and 2nd and 3rd refills. In these relatively long intervals the blood values rose slightly on each occasion. Following the 3rd insufflation, the interval was decreased and the values rose steadily to a maximum.



*Erythropoiesis.* The rather slow increase in erythrocyte concentrations following a period of insufflation suggested at once an increased erythropoietic activity. To this end, reticulocyte counts were made repeatedly on jugular venous blood of all dogs. Entirely negative results were found. At no time did the range of reticulocyte percentages of the pneumothorax period vary from that of the control.

In order to check this unexpected result, the hemoglobin production during the control period was compared with that during insufflations. The method employed was a modification (12) of the classical 'constant level anemia' of Whipple and Robscheit-Robbins (13). Three dogs, one of which was splenectomized, were followed

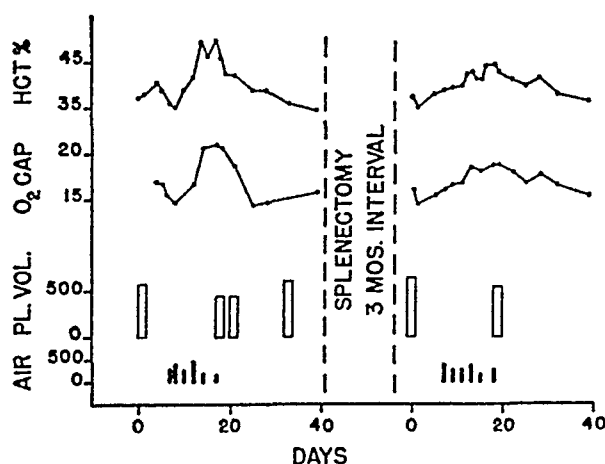
for 4, 4 and 7 months respectively. All animals were bled regularly for 9 weeks in order to exhaust reserve red cells and to establish control hemoglobin production rates. Pneumothorax was then maintained for 21-day periods separated by a control period of similar duration. This process was repeated several times. When comparisons were made of control and pneumothorax periods, two of the dogs showed no

TABLE 1. SUMMARY OF RESULTS FOLLOWING PNEUMOTHORAX

DOG	CONDITION	DURATION OF PNEUMOTHORAX, DAYS	AVERAGE PERCENTAGE CHANGE FROM CONTROL			
			HCT	O <sub>2</sub> CAP	PV	PP
1	Normal	13	+14	+7	-6	+1
2	Normal	11	+24	+27	-20	+6
3	Normal	14	+25	+24	-20	+11
4	Normal	75	+30	+43	-15	0
4	Normal	36	+26	+25	-28	+23
5	Normal	21	+12	+8	-16	0
6	Normal	12	+4		0	0
7	Normal	50	+19	+22		
8	Splenectomized	55	+10	+12	-20	0
2	Splenectomized	12	+15	+16	-13	0
9	Splenectomized	17	+11		-9	0
10	Splenectomized	17	+10		-5	0

HCT = Hematocrit percentage. O<sub>2</sub>CAP = Oxygen capacity (vol. per cent). PV = Plasma volume. PP = Plasma protein percentage.

Fig. 3. EFFECTS OF PNEUMOTHORAX on the same dog before and after splenectomy are shown above. A very definite post-pneumothorax increase on hematocrit percentage and O<sub>2</sub> capacity (vol. per cent) occurred after splenectomy. Plasma volume (cc.) showed an inverse relationship to the other values measured.



significant changes. The third dog, a normal animal, gave evidence of marked hemoconcentration during insufflations but no increase in hemoglobin formation.

*Spleen.* In a further attempt to explain the increased red cell concentration, the possible rôle of splenic contraction was examined. The customary pneumothorax procedure was carried out on 4 dogs 2 to 3 months after splenectomy. Figure 3 presents the results obtained from an animal given insufflations both before and after splenectomy. Following splenectomy, pneumothorax produced an increase in hematocrit percentage and oxygen capacity. The increase in the 4 splenectomized animals

ranged from  $+10$  to  $+15$  per cent of control values. This is a slightly smaller increase than that shown by the larger control series (table 1). The difference between the two groups is of questionable significance, because of the relatively smaller number of splenectomized dogs and the range of individual variation.

*Plasma Volume and Refractive Index.* In order to test the possibility of hemoconcentration, plasma volume measurements were made in 9 dogs, both normal and splenectomized. The results indicated that with one exception plasma volume decreased in all animals following pneumothorax. The average change from control ranged from 0 to  $-28$  per cent. The single animal showing no change was the same one whose hematocrit value increased only  $+4$  per cent over the control figure. The period of maximal elevation of the concentration of blood elements after insufflation coincided with that of minimal plasma volume. Figures 3 and 4 provide examples of

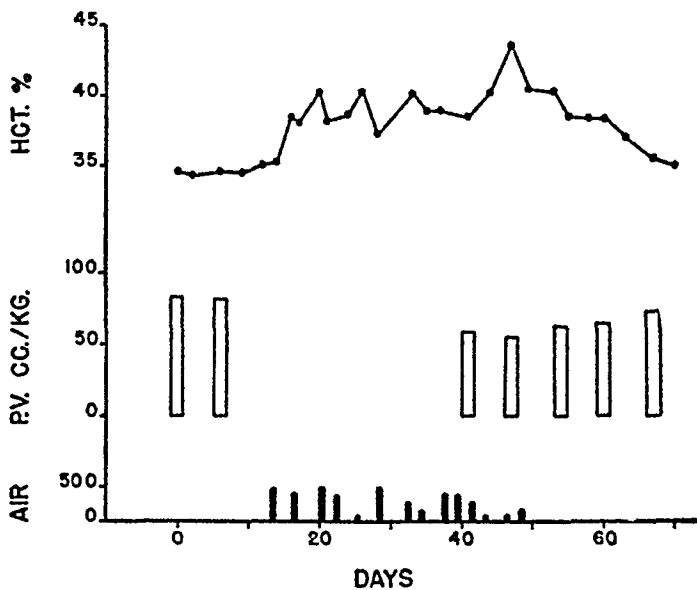


Fig. 4. INFLUENCE OF REPEATED INSUFFLATIONS on hematocrit percentage and plasma volume (cc/kg. body weight) is shown. The inverse relationship between these two values is evident.

the relationship of plasma volume and hematocrit value and table 1 gives a summary of the data.

The number of grams of protein per 100 cc. of plasma was calculated from the refractive index of plasma during plasma volume determinations. Of the 11 series of measurements on 9 dogs, 8 showed no change following pneumothorax. Three, however, increased  $+6$ ,  $+11$ , and  $+23$  per cent, above control values (table 1).

#### DISCUSSION

Any increase in value of the formed elements of the blood following pneumothorax required from 3 to 8 days to become apparent and 2 to 4 weeks to reach a maximum. It is of interest to note an immediate hematocrit elevation has been reported by Henry and his group (14, 15) in both cat and man within 30 minutes during breathing against a resistance of 20 to 120 mm. Hg. Increase in intrathoracic pressures in the present study never exceeded 5 mm. Hg and usually were much less.

The absence of demonstrable reticulocytosis during the phase of increasing hematocrit, oxygen capacity and red blood cell values negates erythropoietic stimulation.

In addition, the failure of periods of pneumothorax to cause an increased hemoglobin production during 'constant anemia' substantiates this conclusion. Earlier workers (1, 2) believed the polycythemic response to be the result of an increased blood cell production caused by anoxic anoxia. Under the conditions of the present study, neither anoxic anoxia nor erythropoietic stimulation was found.

The post-pneumothorax concentration of formed blood elements persisted after removal of the spleen. If splenic contraction plays a part during pneumothorax its influence is negligible.

The decrease in plasma volume appears to be the most likely explanation of the increased hematocrit value in 9 of 10 animals during the insufflation periods as shown in table 1 and an example is presented in figure 4. Direct determination of the circulating red blood cell mass would be desirable but was not performed. Calculation of total blood volume and red blood cell mass from plasma volume and hematocrit percentage was made during the control and pneumothorax periods. The change in total blood volume ranged from +1 to -16 per cent of control values with an average of -10 per cent. Red cell mass values showed a large degree of variation and the change from control ranged from -10 to +25 per cent with an average of +4 per cent.

From the data presented, it would appear that the increased value of the formed blood elements following pneumothorax is largely a manifestation of hemoconcentration. Fluid of unknown composition is lost from the vascular bed under the stress of increased intrathoracic pressure and the changes associated with it. Although some variation existed in the percentage of plasma proteins, the fluid lost from the blood vessels carried protein with it. The site of departure is not known, but the pulmonary capillaries are those subjected to the greatest trauma and therefore likely to permit leakage. On the other hand, the increased intrathoracic pressure might tend to counteract this process.

A difference in the compartmentation of the thoracic cavity of man and dog exists, yet similar increases in hematocrit values have been reported in both species (1-5). Whether all the changes occurring in dogs as a result of pneumothorax take place in man has not been determined. It is of some interest, however, to speculate on the possible rôle of hemoconcentration during therapeutic pneumothorax.

#### SUMMARY

Artificial, closed pneumothorax was maintained in a total of 13 unanesthetized dogs for periods of 11 to 75 days by repeated insufflations of room air. Respiratory compensation was adequate and anoxic anoxia was not present. In a few days the hematocrit, oxygen capacity and red blood cell count values rose above those of the control period and reached a maximum in 2 to 4 weeks. At the end of the pneumothorax period, these values returned to the control level in about a week.

No evidence of erythropoietic stimulation was obtained since reticulocyte percentage and hemoglobin production remained within the control ranges. In addition, the presence of the spleen is not essential to the hematocrit increase. Plasma volumes decreased as the hematocrit values increased and this inverse relationship was main-

tained throughout the course of the procedure. Plasma protein percentage showed no consistent change.

It is concluded that the increased values of the formed elements of the blood during pneumothorax is a result of hemoconcentration caused by loss of fluid from the vascular bed.

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# DEVELOPMENT OF TURBULENCE IN FLOWING BLOOD<sup>1</sup>

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THE conditions which lead to turbulence in flowing blood are of hemodynamic interest for several reasons: *a*) turbulence is generally believed to be an important factor in the production of murmurs; *b*) calculation of the work of the heart depends upon the nature of flow in the central arteries (1); *c*) a study of the development of turbulence yields information important to the understanding of laminar flow in small blood vessels which offer the principal resistance to flow of blood in the peripheral circulation.

Reynolds (2) showed that the transition from laminar to turbulent flow in tubes depends on the dimensionless expression  $Re = \frac{\rho \bar{u} r}{\eta}$  where

$$\begin{aligned} \rho &= \text{density} & \eta &= \text{viscosity} \\ \bar{u} &= \text{average velocity} & r &= \text{radius of tube} \\ Re &= \text{Reynolds' number} \end{aligned}$$

The Reynolds' number represents the ratio of inertial force to viscous force; it is the condition for mechanical similarity of flow around geometrically similar objects. The inertial force per unit volume is proportional to  $\frac{\rho \bar{u}^2}{r}$ , while the viscous force per unit volume is proportional to  $\frac{\eta \bar{u}}{r^2}$ . It is evident that as the flow increases, the inertial force increases faster than the viscous force. The transition from laminar to turbulent flow occurs when the inertial force becomes so great that the viscous force is no longer able to damp stray disturbances in the fluid. When no effort is made to minimize these disturbances in homogenous fluids, this transition occurs at Reynolds' numbers of 1000 to 1100 (critical Reynolds' number).

It has generally been assumed that the flow of blood, like that of homogeneous fluids, would become turbulent at a Reynolds' number of about 1000. Since blood is a heterogeneous system of variable viscosity, this assumption is not justified a priori.

The only experimental measurements of the turbulence point for blood appear to be those of Müller (3) who has employed conventional hydrodynamic methods to determine the critical Reynolds' number for ox blood flowing in glass tubes. Müller has shown that the relations between pressure and flow are such as to indicate a transition from laminar to turbulent flow at Reynolds' numbers in the range 700-1000. Our own experiments were begun without knowledge of Müller's results and were designed to correlate pressure-flow data with a study of cellular orientation during the development of turbulence. We planned to demonstrate the turbulence point in two ways: *a*) by conventional hydrodynamic procedures and *b*) by an electri-

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cal method based on observations of Velick and Gorin (4). These authors observed that the electrical resistance of flowing blood, measured in the direction of flow, was less than that of blood at rest. They showed this phenomenon to be a result of orientation of the cells with their long axes parallel to the flow stream. The non-conducting cells, when oriented, occupy less of the cross-sectional area. They thereby permit more current to flow, much as a Venetian blind, when opened, will admit more light. We decided to measure electrical resistance in the direction of flow simultaneously with pressure and flow, anticipating that electrical resistance would rise when the cellular orientation was disturbed by turbulence.

### METHODS

A diagram of the experimental apparatus is shown in figure 1. Two flow tubes of dimensions shown in the figure were used; each tube was provided with 2 pressure taps, the first tap being placed at a distance of 150 radii from the entrance of the tube in order to obtain 95 per cent of the parabolic velocity distribution for laminar

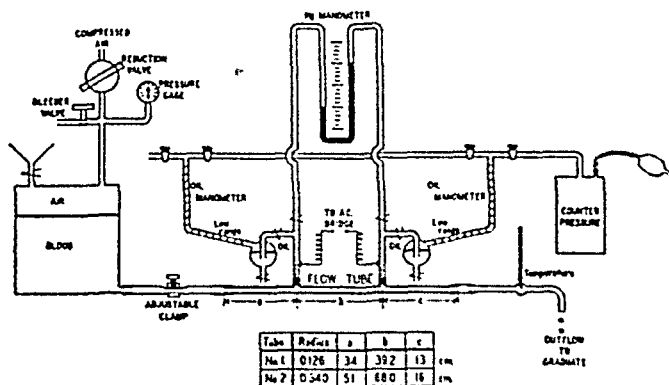


Fig. 1

flow (8). This arrangement eliminated the need for applying kinetic energy corrections. The average radius of each tube was determined by the mercury-weight method and by measuring the electrical resistance, between the pressure taps, of saline of known specific conductivity (5, 6).

The pressure drop between taps was measured by 2 oil manometers at low pressures and a U-tube mercury manometer at high pressures. The oil manometers were provided with inclined scales which could be read to the nearest 0.02 cm. H<sub>2</sub>O over a range of 0 to 1.8 cm. H<sub>2</sub>O. Vertical scales were provided for intermediate pressures. The mercury manometer was used for higher pressures up to 600 mm. Hg: corrections were applied to allow for the weight of saline within the conduits leading to each arm of the U-tube (mercury) manometer. Flows were measured with graduated cylinders and a stop watch. Temperature of the blood was determined from a thermometer inserted near the outflow. Electrical resistance in the axis of flow was measured between platinum electrodes (fig. 1) inserted into the pressure taps. The measurements were made with an A.C. bridge operating at 1 to 3 kc. Reactive components were balanced out capacitatively using an oscilloscope as a null indicator.

The overall hydrodynamic characteristics of the apparatus were checked by measuring the pressure-flow relations of water and computing the viscosity from Poiseuille's Law. The flow of water became turbulent at a Reynolds' number of  $1080 \pm 40$ .

Bovine blood was obtained from the local abattoir, isotonic sodium citrate and heparin being used as anti-coagulants. NaCN (about 1 mM/l.) was added to inhibit oxidative metabolism and thus prevent reduction of the hemoglobin. It is our impression that the cyanide acted as a preservative and delayed hemolysis. Before each measurement the pressure reservoir was shaken to mix the blood; settling of the red cells at zero flow was detectable as a slow progressive increase in electrical conductivity. Ordinarily this settling was negligible even at low flows; samples taken from the outflow at intervals during each experiment showed no significant changes in hematocrit.

### RESULTS

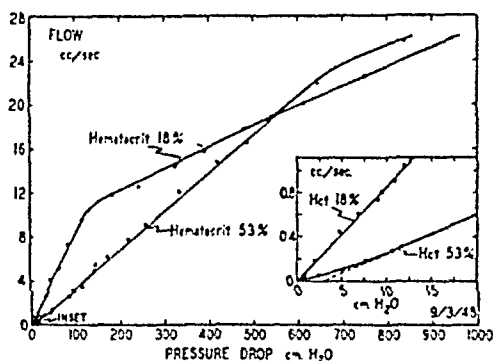
At low flows the apparent viscosity, calculated from Poiseuille's Law, was found to decrease with increasing flow. This anomalous effect is discernible in the inset of figure 2 as a convexity of the pressure-flow curve toward the pressure axis; it is shown in detail in figure 3 for bloods of different hematocrits. It is clear from these results that anomalous flow occurs in large tubes as well as in capillary tubes in which the phenomenon was first demonstrated by Hess (7). At intermediate and high flows the apparent viscosity approached a constant value (fig. 3).

Turbulence was indicated hydrodynamically by a pronounced bend in the pressure-flow curve as seen in figure 2. A more sensitive index of the transition point may be obtained by plotting the dimensionless friction coefficient,  $\lambda = \frac{\Delta P}{\frac{1}{2}\rho\bar{u}^2} \cdot \frac{r}{l}$  against the apparent Reynolds' number on a logarithmic scale as shown in figure 4. With this method of analysis all points fall on the same straight line, regardless of the nature of the fluid or the dimensions of the tube, so long as laminar flow exists. At the turbulence point there is a sharp upward deflection of the curve following which all points fall on a second straight line characteristic of turbulent flow (8). It is seen that the transition point for blood occurred at an apparent Reynolds' number of approximately 1000 for both tubes in the 2 examples illustrated in figure 4. The results of all our experiments with bloods of different temperatures and hematocrits are summarized in table 1. The critical apparent Reynolds' number averaged  $970 \pm 80$ .

The effects of temperature on the pressure-flow relations of blood of constant hematocrit are illustrated in figure 5. It is seen that the flow at which turbulence begins progressively increases with reduction in temperature as would be predicted from the increased viscosity. This results in a curious paradox: it is seen that in certain pressure ranges above turbulence the flow of cold blood is greater at a given pressure than that of warm blood. A similar condition exists in bloods of different hematocrit; thus, blood of high hematocrit may present less resistance to flow than blood of low hematocrit (e.g. in the upper pressure ranges illustrated in figure 2). This paradoxical behavior may be of physiological significance in the turbulent

flow of blood through stenotic apertures. In this case a high hematocrit might actually be advantageous in minimizing resistance to blood flow through the stenosed region.

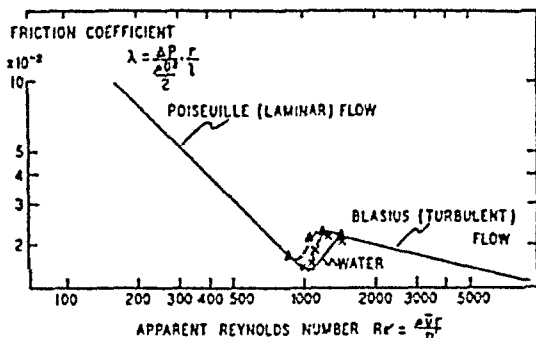
The electrical resistance decreased with increasing flow at low Reynolds' numbers paralleling the changes in apparent viscosity in this range. (Compare figs. 3 and 6).



PRESSURE — FLOW DATA

SHOWING ANOMALOUS VISCOSITY AND DEVELOPMENT OF TURBULENCE  
IN BOVINE BLOOD AT 27°C

$r = 0.126$  cm  $l = 39.2$  cm

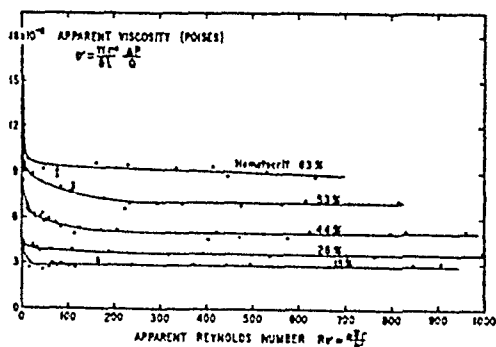


DEVELOPMENT OF TURBULENCE IN FLOWING BLOOD

Bovine blood, hematocrit 29% at  $29 \pm 1^\circ$  C

x — Tube No. 1.  $r = 0.126$  cm  $l = 39.2$  cm

Δ — Tube No. 2.  $r = 0.340$  cm  $l = 68.0$  cm



VARIATION OF BLOOD VISCOSITY WITH REYNOLDS' NUMBER

Bovine blood at  $30 \pm 4^\circ$  C  $r = 0.126$  cm  $l = 39.2$  cm

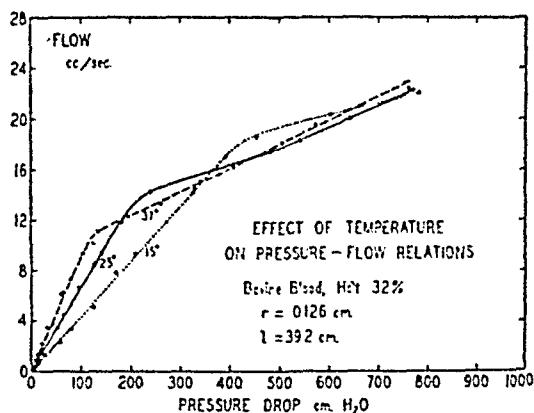


Fig. 2 (upper left). PRESSURE-FLOW DATA showing anomalous viscosity and development of turbulence in bovine blood at  $27^\circ$  C.;  $r = 0.126$  cm.,  $l = 39.2$  cm.

Fig. 3 (lower left). VARIATION OF BLOOD VISCOSITY with Reynolds' number. Bovine blood at  $30 \pm 4^\circ$  C.;  $r = 0.126$  cm.,  $l = 39.2$  cm.

Fig. 4 (upper right). DEVELOPMENT OF TURBULENCE in flowing blood. Bovine blood, hematocrit 29% at  $29 \pm 1^\circ$  C.

x — Tube No. 1.  $r = 0.126$  cm.,  $l = 39.2$  cm.

Δ — Tube No. 2.  $r = 0.340$  cm.,  $l = 68.0$  cm.

Fig. 5 (lower right). EFFECT OF TEMPERATURE on pressure-flow relations. Bovine blood, hematocrit 32%;  $r = 0.126$  cm.,  $l = 39.2$  cm.

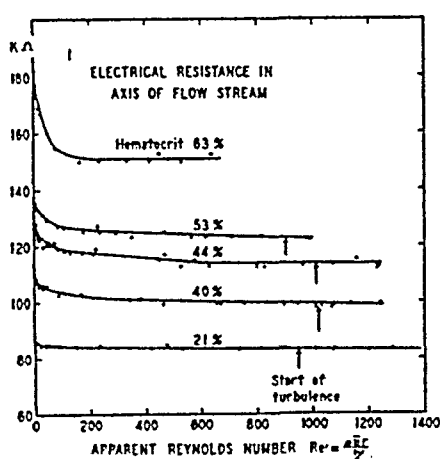
Presumably these changes in electrical resistance result primarily from orientation of the cells in the flow stream. At zero flow the (non-conducting) cells are randomly oriented and the electrical resistance is high; as flow progresses, the cells orient to present least hydrodynamic resistance and both the apparent viscosity and the electrical resistance diminish in regular fashion toward constant values.

At the turbulence point the electrical resistance did not rise but, contrary to expectation, remained constant up to a Reynolds' number of 2500 which was the

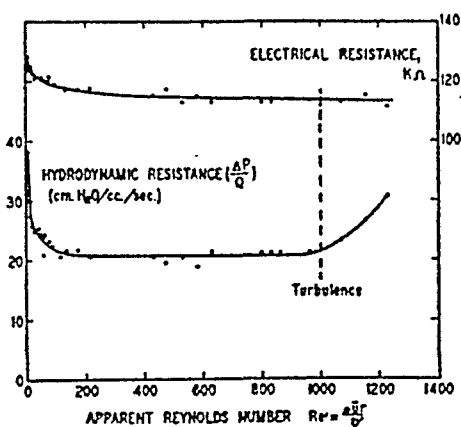
highest we could conveniently obtain with our apparatus. This is shown in figure 6 for bloods of various hematocrits. The contrast in behavior at turbulence between

TABLE 1. CRITICAL APPARENT REYNOLDS' NUMBERS OF BOVINE BLOOD IN GLASS TUBES OF DIFFERENT BORE AND AT DIFFERENT TEMPERATURES AND HEMATOCRITS

TUBE RADIUS $r$	HEMATOCRIT	DENSITY $\rho$	TEMPERATURE	APPARENT VISCOSITY JUST BELOW TURBULENCE $\eta$ (poises)	CRITICAL APPARENT REYNOLDS' NUMBERS $\frac{\rho \bar{u} r}{\eta}$
cm.	%	gm/cc.	°C.		
.126	18	1.033	27	.029	900-1090
.126	21	1.035	24	.033	890-1010
.126	28	1.043	29	.037	1000-1090
.126	40	1.048	34	.047	1015-1035
.126	44	1.054	27	.051	960-1070
.126	53	1.054	28	.070	820-990
.126	32	1.040	14	.057	815-945
.126	32	1.045	26	.036	850-1050
.126	32	1.048	37	.026	960-1140
.340	18	1.032	27	.030	845-1000
.340	29	1.042	29	.040	785-1110
.340	45	1.051	28	.047	880-1080
Mean					970 $\pm$ 80



ELECTRICAL RESISTANCE  
OF FLOWING BLOOD  
Bovine blood,  $t = 30 \pm 4^\circ\text{C}$   
Tube  $r = 0.126$  cm.  $l = 39.2$  cm.



HYDRODYNAMIC AND ELECTRICAL RESISTANCE  
OF FLOWING BLOOD

Bovine blood 44% hematocrit  
 $T = 27^\circ\text{C}$   $r = 0.126$  cm.  $l = 39.2$  cm.

Fig. 6 (left). ELECTRICAL RESISTANCE of flowing blood. Bovine blood, at  $30 \pm 4^\circ\text{C}$ ;  $r = 0.126$  cm.,  $l = 39.2$  cm.

Fig. 7 (right). HYDRODYNAMIC AND ELECTRICAL RESISTANCE of flowing blood. Bovine blood, 44% hematocrit; at  $27^\circ\text{C}$ ;  $r = 0.126$  cm.,  $l = 39.2$  cm.

hydrodynamic resistance, defined as  $\frac{\Delta P}{Q}$ , and electrical resistance is clearly shown in figure 7. We can only conclude that the red cells remain oriented despite the turbulence demonstrated simultaneously by hydrodynamic methods.

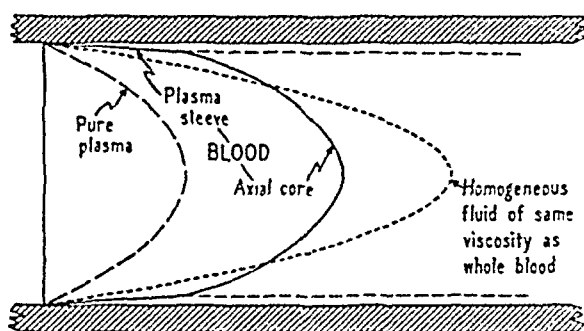
## DISCUSSION

The constancy of electrical resistance of blood in which an orientation effect is electrically demonstrable at low flows indicates that the cells remain oriented in turbulent flow. It is difficult to see how they could remain oriented if they did not occupy a region of the flow stream which was free of turbulence. It follows, then, that turbulence must occur in a region which is free of cells.

It is well known that, in vessels of small caliber, the cells tend to accumulate in the axial stream (9, 10). Presumably this same phenomenon occurs in larger vessels. Surrounding the axial core of cells and plasma is a 'peripheral sleeve' of essentially cell-free plasma. The constancy of electrical resistance at turbulence indicates that laminar flow is maintained in the axial core, and that turbulence is confined to the peripheral sleeve.

If this hypothesis is correct, we may think of the apparent viscosity of blood in laminar flow as the resultant of two viscous resistances in parallel: a) the viscosity of plasma alone in the peripheral sleeve and b) the viscosity of cells plus plasma in the

Fig. 8. VELOCITY DISTRIBUTION CURVES just before transition to turbulent flow. The velocity distribution curve for this blood was calculated on the assumption that turbulence occurred when the average velocity of the peripheral sleeve plasma equalled the average velocity of plasma alone at turbulence in the same tube. If this assumption be accepted then the values of radius and viscosity of the axial core can be calculated from the observed viscosities of whole blood and plasma; substitution of these values in Newton's laws of viscous flow yield the composite velocity distribution curve shown.



axial core. This is represented in figure 8 for laminar flow just below the transition to turbulence. As flow is further increased, a critical average velocity of the peripheral plasma is eventually reached beyond which the inertial force in the peripheral sleeve alone is sufficient to maintain turbulence. In the axial core, however, the viscous stabilizing influence of the red cells is still great enough to prevent this turbulence from spreading beyond the peripheral sleeve. We thus have a two-phase system, with turbulence in the (thin) peripheral sleeve proceeding simultaneously with stabilized viscous flow in the central core.

The hypothesis that blood flows as a two-phase system has several implications of theoretical and practical importance:

1. Application of Newton's Law of Viscosity to this two-phase system yields the relation  $\eta' = \frac{\mu}{1 - \alpha^2 \left(1 - \frac{\mu}{\eta_0}\right)}$  where  $\eta'$  = apparent viscosity of whole blood;

$\mu$  = viscosity of plasma;  $\eta_0$  = viscosity of central core, and  $\alpha$  = fraction of cross-sectional area of tube occupied by the central core. The value of  $\alpha$  at turbulence has not been established with certainty but it is probably not less than 0.85 under the

conditions of our experiments (see legend to fig. 8). This estimate yields a minimum value for  $\eta_0$  which is about 10 times the viscosity of plasma.

2. Doubt is cast on the technique used by Ralston and Taylor (1, 11) in determining the character of flow in glass or lucite tubes inserted into the aortas of dogs and cats. These authors injected India ink into the left ventricle or proximal aorta, and observed the appearance of streamlined filaments of ink in the tubes. This conclusion, valid for water, is not justified for blood in which core flow may be laminar while peripheral sleeve flow is turbulent. From the point of view of calculating the kinetic work of the heart it is clear from figure 8 that the velocity distribution of blood flow cannot be considered a continuous parabolic distribution based upon its apparent viscosity as would be justifiable in a homogeneous fluid.

3. The concept of flowing blood as a two-phase system may have an important bearing on the problem of peripheral resistance. The energy required to maintain laminar flow of homogeneous fluids is greatest in the periphery where the rate of shear is highest. In a two-phase system where the axial core viscosity is considerably greater than that of the peripheral sleeve, this difference between peripheral and central rates of shear is greatly accentuated (fig. 8). It would appear, therefore, that in a two-phase system, most of the energy required to maintain flow is dissipated in the peripheral sleeve, the axial core being carried along somewhat like a log in the center of a stream. It is suggested that this pattern of energy dissipation may apply to blood flowing in the arterioles which present the major resistance to flow of blood in the peripheral circulation.

#### SUMMARY

The development of turbulence in bovine blood flowing through medium bore glass tubes is described.

Turbulence was found by conventional hydrodynamic methods to occur at an apparent Reynolds' number of  $970 \pm 80$ . This value was independent of tube size, concentration of red cells and of temperature (table 1). At very small Reynolds' numbers the apparent viscosity decreases with increasing flow (fig. 3). Anomalous flow of blood is therefore not restricted to capillary tubes but is detectable, with refined methods, in large tubes at low rates of shear.

Electrical resistance, measured in the axis of flow, was found to diminish with flow in a regular manner paralleling the variation in apparent viscosity (figs. 6 and 7). Evidently the non-conducting cells, in orienting to diminish hydrodynamic resistance to flow, also present less cross-sectional area opposing the flow of electric current. Contrary to expectation, the electrical resistance remains unaltered by the establishment of turbulent flow (figs. 6 and 7) even at Reynolds' numbers up to 2500. This suggests that the cells remain oriented and that turbulence occurs in a region free from cells.

The facts are consistent with the hypothesis that blood flows as a two-phase system comprising a peripheral plasma sleeve and a central core of plasma plus cells (fig. 8). Turbulence may develop in the peripheral plasma phase while laminar flow and cellular orientation continue undisturbed in the axial core. The results are discussed with reference to *a*) calculation of the kinetic work of the heart, *b*) the

flow of blood through stenotic apertures and c) the nature of flow in the peripheral arterioles.

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## METHOD FOR CONTINUOUS INTRAVENOUS ADMINISTRATION OF NUTRITIVE SOLUTIONS SUITABLE FOR PROLONGED METABOLIC STUDIES IN DOGS<sup>1, 2</sup>

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IN A study of the nitrogen metabolism of dogs fed by vein, hypertonic glucose and glucose-protein solutions were administered continuously for as long as 141 days, by the use of a plastic tube extending into the superior vena cava. Hypertonic solutions were employed so that the total fluid input would approximate the amount ingested normally by animals eating *ad libitum*.

The Stengel-Vars apparatus (1), which permitted normal and free movement of the dog in a metabolism cage, was satisfactory in our hands when physiologic concentrations of glucose and saline were used, but solutions of 50 per cent glucose and hypertonic glucose-protein caused mechanical and technical difficulties. The Jacobs (2) syringe-swivel unit would occasionally stick or freeze and offered a favorable site for bacterial contamination. The volume of the system, from the pump to the indwelling catheter was large (ca. 30 ml.) and thermal reactions were frequent.

The technique and apparatus evolved utilized pertinent parts and principles of the initial set-up and, in the main, consisted of a pump, flexible tubing circuit and counter-weight system.

A scheme is illustrated in figure 1 and a photograph in figure 2. The solution

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<sup>1</sup> These studies were supported in part by the Knox Gelatine Protein Products Inc., Camden, N. J.

<sup>2</sup> A preliminary report of this work was made at the Annual Clinical Congress of the American College of Surgeons, New York City, September 10, 1947, *Bull. Am. Coll. Surg.* 32:255, 1947.

bottle, with a cotton air intake filter, was connected to the Debakey pump tube with a hypodermic needle. A modified Debakey type pump was driven by a constant speed A.C. synchronous motor through 2 permanent gear trains (33:12:1 and 6:1) and an adjustable train containing 2 replaceable gears. The desired output speed was obtained by proper selection of the variable gears. The fluid delivered by one tube could be varied from 3.5 to 30.0 ml/hour and with 3 pumping tubes in simultaneous use up to 90.0 ml/hour could be delivered.

A wire mesh filter<sup>3</sup> enclosed in a glass tube and a 4-inch segment of readily distensible latex tubing were incorporated into the pump tube outlet. The latter served as a balloon or bubble within which the fluid could accumulate if the capillary system became obstructed. In addition, a length of one-inch Penrose tubing encased the latex segment in the event of a leak or rupture.

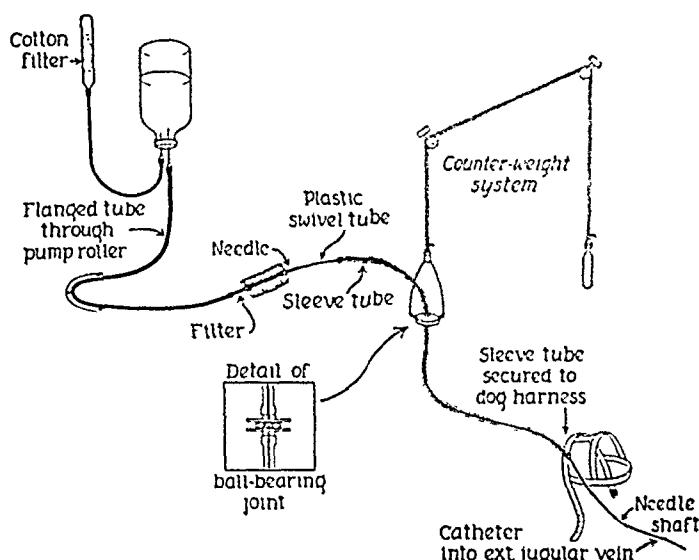


Fig. 1. SCHEMATIC DRAWING of component parts for continuous intravenous injection of fluids into ambulatory animals.

A 6-foot length of 'swivel' tubing<sup>4</sup> was connected to the pump tube outlet by a hypodermic needle and to the intravenous catheter by a needle shaft.

Two segments of a larger, plastic, 'sleeve' tube<sup>5</sup> housed and protected the swivel tube. A ball-bearing joint was inserted between the segments to prevent kinking with rotation of the dog. A short segment was attached to and extended several inches above the ball-bearing joint. A longer segment extended from the joint downward through a hole in the cage top to the harness. Vertical motion was accomplished by a simple counterweight system attached to the bearing and harness. The full length of the swivel tube was maintained by securing it with tape to the upper and lower free ends of the sleeve tube.

All motion of the dog, both rotary and vertical, was transmitted through the swivel tube. This plastic tube of polyvinyl chloride<sup>6</sup> is relatively soft, elastic, flexible

<sup>3</sup> Obtained from Army-Navy blood plasma sets.

<sup>4</sup> Irvington Varnish and Insulator Co. Irvington, N. J.—0.022", i.d., 0.054", o.d.

<sup>5</sup> Irvington Varnish and Insulator Co.—0.125", i.d., 0.25", o.d.

<sup>6</sup> Irvington Varnish and Insulator Co.—0.022", i.d., 0.054", o.d.

and nonwetable. A 6-foot length with both ends secured could be twisted or rotated more than 100 times in one direction without occluding the lumen providing the original length was maintained. A longitudinal pin stripe or line was painted on in order to judge the degree of rotation. Untwisting was performed when necessary. The capacity of this capillary tubing is about 0.1 cc/foot, and consequently the movement of a given unit of solution was relatively rapid with pump outputs of only 10.0 to 16.0 cc/hour. Thus, accidental introduction of bacteria into the fluid column when

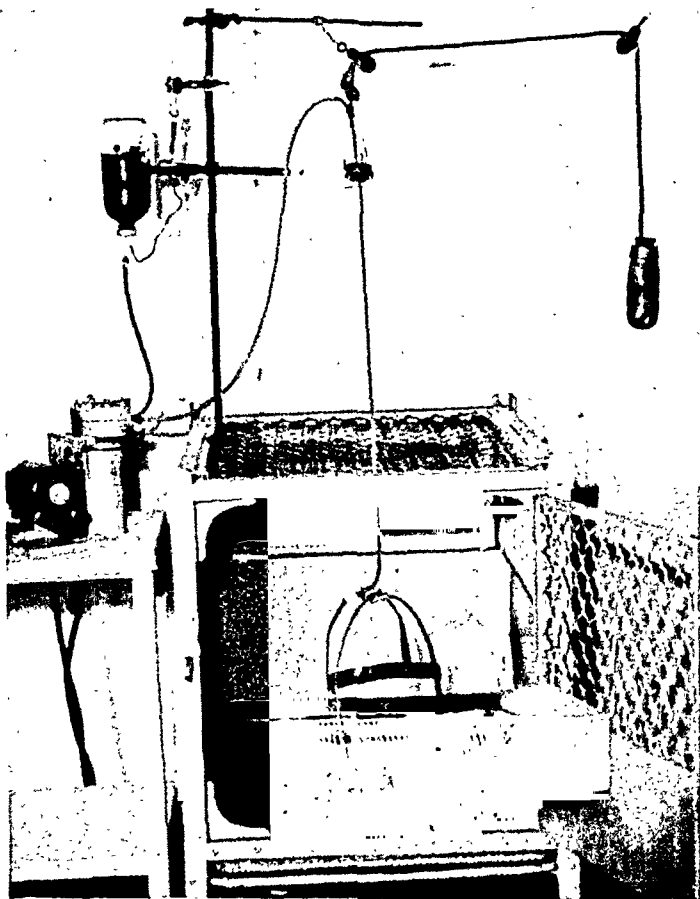


Fig. 2. PHOTOGRAPH OF EQUIPMENT set up for continuous intravenous injection of fluids into dogs. The Penrose-tubing cover, described in the text, is not present in this photograph.

joining the apparatus to the intravenous catheter did not result in gross contamination of the system.

Venous catheterization, as described by Zimmerman (3), was performed by threading another plastic capillary tube through a hypodermic needle, previously inserted into the external jugular vein, until the tip was in the superior vena cava. The needle was withdrawn and local pressure was applied for hemostasis. The vein was not ligated. A thin patch of gauze and collodion anchored the tube to the skin. The outer end of the catheter was joined to the swivel tube with a needle shaft and taped to the harness. A muslin or canvas jacket covered the harness and the neck of the dog.

The tip of the indwelling venous catheter was placed in the superior vena cava so that greater dilution of the hypertonic infusions occurred. The rates of infusion

were about 0.2 to 0.3 ml/minute whereas the cardiac output of a 10-kg. dog is about 2000 ml/minute. In earlier experiments, specimens of caval blood were withdrawn through a second tube, the tip of which was inserted one inch distal to that of the infusion catheter. Blood glucose analyses were normal.

The plastic catheters, polyvinyl chloride and polyethylene<sup>7</sup>, ranged in outside diameter from 0.024" to 0.054" (equivalent to o.d. of 21- to 17-gauge needles) and could be threaded through 13- to 18-gauge hypodermic needles. The catheters were sterilized by immersing in an aqueous solution of Zephiran (1:1000). Polyethylene tubes may be boiled, but not autoclaved.

The fluid circuit, DeBakey pump tube, latex tube and swivel tube were sterilized as a single unit by autoclaving. Care was taken to avoid pressure defects in the plastic tubing during the heating process.

The diets consisted of 50 per cent glucose, Amigen<sup>8</sup>, and/or gelatin Knox P-20 in hypertonic glucose, which were administered continuously by vein. A multiple vitamin mixture (JH47-10210)<sup>9</sup>, McCollum's 185 salt mixture, and water *ad libitum* were given orally.

Since this technique and apparatus were used in metabolic experiments, the dogs were not killed and comprehensive pathologic studies were not undertaken.

Table 1 summarizes the experience to date. The same veins have frequently been re-employed in subsequent experiments. Multiple use, however, often caused thickening and obliteration of the lumen. Rarely did infection appear in the skin or subcutaneous tissues surrounding the intravenous catheter.

Edema or swelling of the ventral aspect of the neck associated with fever occurred in 4 animals (*dogs 911, 1045, 239, 44*) after 29, 24, 46 and 141 days respectively of the third or fourth experiment. Three recovered upon removal of the tube. *Dog 239* died 12 days after the completion of the third experiment, with massive pleural effusions. At postmortem the polyvinyl chloride catheter, which had been used in the second experiment (67 days) and not removed, was noted in the right external jugular vein, superior vena cava, auricle and ventricle. The physical properties of this plastic had changed; the softness and elasticity were no longer present. The wall of the superior vena cava was markedly thickened in one area with a local thrombophlebitic process into which the tube was incorporated, just as the cava curves or angulates to enter the auricle. Two months after completion of the third experiment *dog 564* was electively killed. Pleural effusions and markedly thickened superior vena cava were evident. *Dog 144* was killed two weeks after the fourth experiment (141 days). Both jugular veins and the superior vena cava were patent and contained no obvious areas of thrombophlebitis. The work of Cresson and Glenn (4) and Dr. Paul Nemir, of this laboratory, has given us the impression that the tube per se played the major role when vessel alteration was encountered. Limited experience indicated a high incidence of vessel damage and occlusion when the small peripheral veins were catheterized. The volume of blood flow and cross sectional area of these veins were small in contrast to those of the external jugular veins and superior vena

<sup>7</sup> Surprenant Electrical Insulation Company, Boston, Mass.

<sup>8</sup> Kindly supplied by Dr. W. M. Cox, Jr., Mead Johnson & Co.

<sup>9</sup> Kindly supplied by Mr. Lee Caldwell, Eli Lilly & Co.

TABLE 1. EXPERIENCE WITH INTRAVENOUS PLASTIC CATHETERS USED IN INFUSION OF HYPERTONIC SOLUTIONS INTO SUPERIOR VENA CAVA OF DOGS

DOG NO.	EXPER.	DATE	JUGULAR USED	TUBE <sup>1</sup> , DAYS	50% <sup>2</sup> SOLUTION, DAYS	TOTAL DAYS	
						Tube	50% Sol.
1039	1	10-18-46	R	12	10		
	2	11-22-46	L	29			
	3	1- 6-47	R	3		66	28
	4	3-20-47	R	11	7		
	5	8- 1-47	L	11	11		
911	1	11- 8-46	R	29	7		
	2	12- 7-46	L	16	9		
	3	1- 7-47	R	4		65	32
	4 <sup>3</sup>	3-28-47	R	16	16		
100	1	4-23-47	R	24	11		
	2	11-12-47	R	26	26	50	37
1045	1	1-10-47	R	18	18		
	2	3-28-47	R	39	39	76	76
	3 <sup>3</sup>	7-14-47	R	19	19		
239	1	2-25-47	R	29	27		
	2	5- 6-47	R	11	11	80	78
	3 <sup>3</sup>	5-26-47	L	40	40		
908	1	1-28-47	R	5	5		
	2	2-26-47	L	29	29	54	45
	3	5- 6-47	L	20	11		
564	1	2-11-47	R	37	14		
	2	7-30-47	L	8	8	53	28
	3	8- 7-47	R	8	6		
44	1	1-17-47	R	35	15		
	2	6-26-47	L	34	34		
	3	1- 7-48	L	137	137	209	189
	4 <sup>3</sup>	5-26-48	R	3	3		
565	1	3-24-47	R	54	4		
	2	5- 7-47	R	10	10	97	47
	3	6-26-47	L	33	33		
907	1	2-12-47	R	7	7		
	2	2-19-47	L	20	6	70	56
	3	5-26-47	L	43	43		
144	1	8-11-47	R	17	15		
	2	10-29-47	L	56	56	123	126
	3	4- 9-48	L	55	55		
181	1	12- 3-47	R	84	84	84	84
232	1	3-19-48	R	27	27	27	27
189	1	4-15-48	R	50	50	50	50

<sup>1</sup> Tube days refers to the period of time the catheter was *in situ*, whether being used for infusions or not.

<sup>2</sup> Solution days refers to the actual period of time infusions were performed.

<sup>3</sup> Experiment terminated with clinical signs of vascular abnormality.

cava. No signs or indications of gross caval pathology were noted in any of these dogs during the first or second metabolic experiment.

This technique and apparatus permitted free movement of the dog in the metabolism cage while receiving continuous injections of hypertonic glucose and glucose-protein solutions during metabolic studies. Catheterization of the bladder and withdrawal of blood specimens were carried out without interruption to the infusions.

The dogs so infused for periods of 4 to 20 weeks appear normally active and in good physical condition except for the above-mentioned complications.

We wish to acknowledge the valuable advice and assistance of Dr. Lyle Peterson and Mr. James Walker.

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# NITROGEN BALANCES OF DOGS CONTINUOUSLY INFUSED WITH 50 PER CENT GLUCOSE AND PROTEIN PREPARATIONS<sup>1, 2</sup>

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IN THE experiments to be presented, nitrogen metabolism was studied in dogs which received the majority of food stuffs by vein. Hypertonic solutions of glucose were infused continuously in order to supply adequate non-protein calories for energy needs and at the same time remain within the physiologic range of fluid input. A study of the nitrogen metabolism during the administration of glucose with protein hydrolyzates and gelatin P-20 in post-operative patients has been reported by Riegel *et al.* (1).

## METHODS AND MATERIALS

The solutions were injected into the superior vena cava by means of an indwelling plastic capillary catheter and an injecting apparatus which permitted free movement of the dog in the metabolism cage (2, 3). Fifty per cent glucose was injected at a rate of approximately 25 cc/kg. of initial body weight per day. During periods of protein administration, the amounts of infused nitrogen were comparable and equivalent calories of glucose were utilized. The infused fluid volumes were constant except when mechanical difficulties arose.

A multiple vitamin mixture (0.5 cc/kg.)<sup>3</sup> and McCollum's 185 salt mixture (0.25 gm/kg.) were given orally each day and water was allowed *ad libitum*. Amigen,<sup>4</sup> an enzymatic hydrolyzate of casein, and gelatin-Knox P-20<sup>5</sup> were the protein preparations used. Gelatin P-20 has an average molecular weight of about 35,000 and 99 per cent of the nitrogen is precipitable with tungstic acid. The 'standard' amount of nitrogen, 120 mg/kg. of initial body weight per day, was administered in

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<sup>2</sup> These studies were supported in part by a grant from Knox Gelatine Protein Products, Inc., Camden, N. J.

<sup>3</sup> Kindly supplied by Dr. Lee Caldwell, Eli Lilly and Company (JH47-10210). Each 0.5 cc. contains: 250 units vitamin A; 25 units vitamin D; 0.3 mg. thiamine hydrochloride, 0.3 mg. riboflavin; 0.25 mg. pyridoxine hydrochloride; 0.25 mg. pantothenic acid; 2.5 mg. nicotinamide; 2.5 mg. inositol; 2.5 mg. para-aminobenzoic acid; 0.05 mg. 2 methyl 1,4 naphthoquinone; 2.5 mg. ascorbic acid; 50 mg. ricebran concentrate; 25 mg. linoleic acid; 2.5 mg. distilled natural tocopherols; 15 mg. choline chloride.

<sup>4</sup> Kindly supplied by Dr. W. M. Cox, Jr., Mead Johnson Company.

<sup>5</sup> Kindly furnished by Dr. Dee Tourtellote, Knox Gelatine Protein Products, Inc.

most experiments as: 1) amigen alone, 2) gelatin P-20 alone, 3) amigen and gelatin P-20 in equal amounts, 4) gelatin P-20 supplemented with 2.0 mg. of DL-methionine and 1.0 mg. of DL-tryptophane nitrogen/kg/day. In a few single experiments solutions containing  $\frac{1}{2}$ , 2 or 3 times the 'standard' amounts of amigen or gelatin nitrogen were infused. Also a comparison was made of nitrogen balance during continuous intravenous and continuous intragastric injection.

The total nitrogen in urine and plasma was determined by micro Kjeldahl digestion and titration. Urine urea and ammonia nitrogen were analyzed by the aeration method of Van Slyke and Cullen (4). The differential precipitation of plasma

TABLE 1. NITROGEN EXCRETION DURING INFUSION OF 50 PER CENT GLUCOSE AND 50 PER CENT GLUCOSE SUPPLEMENTED WITH 2 MG. DL-METHIONINE PLUS 1 MG. DL-TRYPTOPHANE-NITROGEN PER KG. PER DAY

DOG NO. <sup>1</sup>	URINARY		NITROGEN		50% GLUCOSE I.V.	
	6-9 days		10-13 days		cal/kg/day	gm/m <sup>2</sup> /hr.
	mg/kg	gm/m <sup>2</sup>	mg/kg.	gm/m <sup>2</sup>		
239	96	2.36	86	2.13	45	12.3
	100	2.45			48	13.0
908	105	2.58	109	2.68	45	12.2
907	111	2.90			42	12.3
44	113	2.58			51	12.8
911	119	2.85	110	2.65	48	12.0
1045	130	3.22	104	2.56	44	12.0
189	133	3.00	114	2.67	48	12.5
144	127	2.78	131	2.88	51	12.3
144	131	2.88			41	11.6
					50% Glucose with meth. tryp.	
44	73	1.75			47	12.5
181	79	1.63			42	11.7

<sup>1</sup> Experiments on 239, 44, 144, 6 months apart.

gelatin, native plasma and protein precipitable urine gelatin was carried out with a modification of the method described by Waters (5).

Male and female dogs of 10 to 16 kg. in weight were used in these experiments after preparation with distemper prophylaxis and effectual therapy for infestation by intestinal parasites.<sup>6</sup> The initial weight was obtained 18 hours after the last feeding and the subsequent ones at the end of each period. The dogs were catheterized every 2 days during the experiment and separate analyses were made of each 48-hour pooled specimen. Stools were quite infrequent after the fifth day. Fecal nitrogen was not determined.

Dogs considered to be normal and in a good state of nutrition were infused initially with the 50 per cent glucose, protein-free diet for 9 to 13 days to establish

<sup>6</sup> We wish to thank Dr. Mark Allam, University of Pennsylvania School of Veterinary Medicine, for his cooperation.

the level of basal nitrogen excretion. Following such a regimen, the different protein preparations in hypertonic glucose solution were injected for periods of varying duration, one series of dogs for about one week, the other for 2 to 3 weeks or longer.

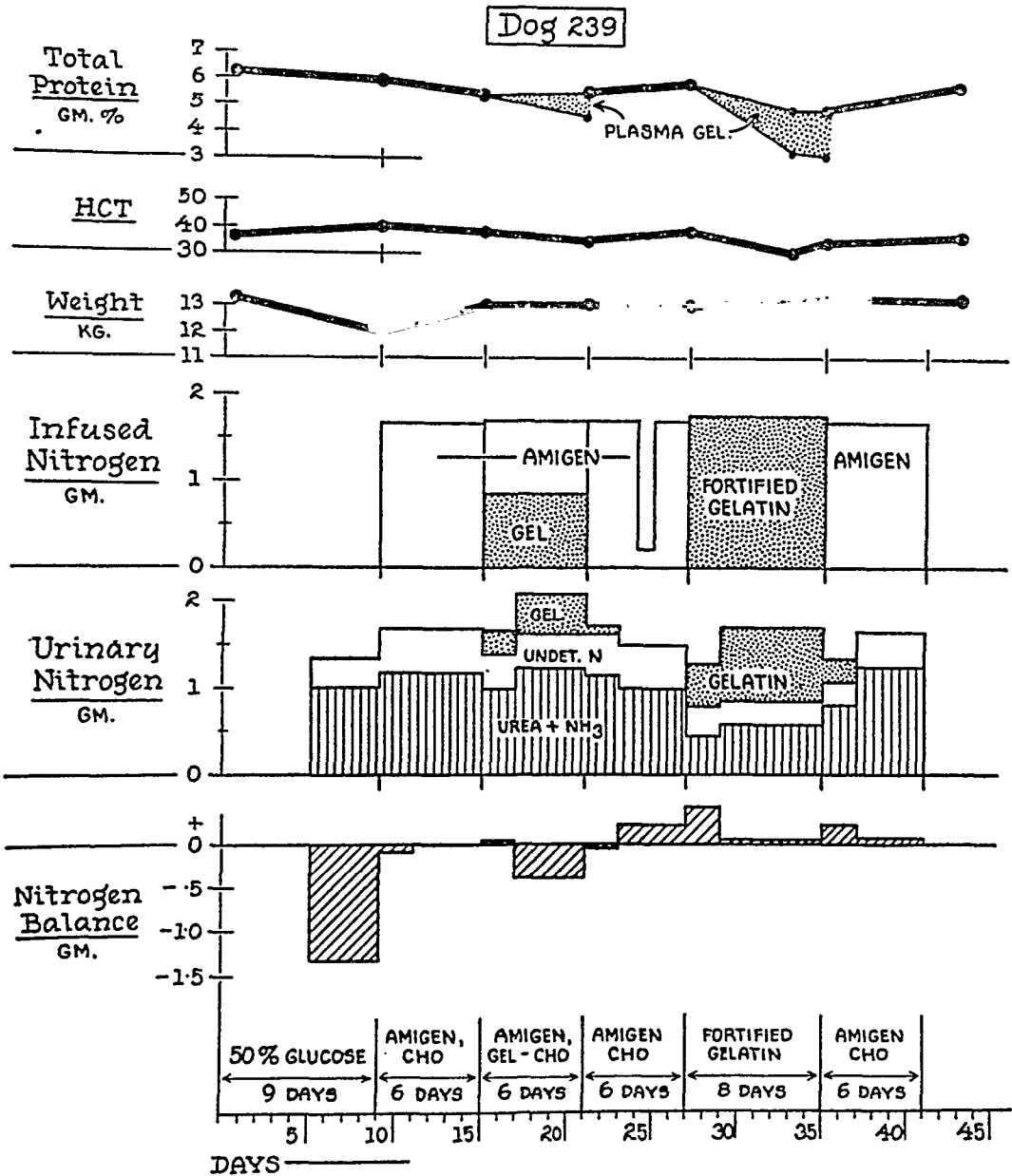


Fig. 1. NITROGEN BALANCES during infusions of different protein preparations following one another. (Lowered nitrogen input on 25th day was due to technical difficulties.)

RESULTS

The values for nitrogen excretion during the initial 6th- through 13th-day period of 50 per cent glucose, protein-free administration are illustrated in table 1. The average outputs of different dogs varied considerably during the 6 to 9-day period, but a maximal level of 114 mg/kg/day was reached during the 10th- through 13th-day period, with the exception of *dog 144*. Two dogs were infused with 50 per cent

glucose containing methionine and tryptophane in amounts previously stated. A diminished nitrogen output was evident.

In one group of 3 dogs the infusion of different types of protein solutions followed one another. A representative example is illustrated in figure 1. Urinary nitrogen and nitrogen balance values are given as period averages except for the first two days of the gelatin P-20 infusions and the subsequent infusions, so as to indicate the delayed response in excretion of both the macromolecular gelatin and other urinary nitrogen. The average excretion of the colloidal gelatin was approximately 50 per cent of that infused and it was included in computing the nitrogen balances.

The nitrogen balance during the infusion of the amigen-gelatin P-20 was not as favorable as with amigen alone. An intermediate balance was obtained with the gelatin P-20 fortified with methionine and tryptophane, but there was a marked decrease in the urea and ammonia nitrogen fraction. A tendency toward a more positive nitrogen balance was observed during the second and third amigen periods and probably reflected progressive protein depletion of the dog.

The average maximum concentrations of plasma gelatin of these dogs were 0.7 gm. per cent and 1.5 gm. per cent at the end of the amigen-gelatin and 'standard' gelatin infusions. The native plasma protein concentrations were proportionally depressed so that little alteration of the total plasma colloid occurred when smaller amounts of gelatin were given. Some hemodilution appeared with the larger quantities.

To obviate the immediate influence of the metabolism of one protein preparation upon a succeeding one, the protein administration in a second group of dogs was preceded and followed by a protein-free base-period. Typical examples of this group are illustrated in figures 2 and 3.

The nitrogen excretion values in *dog 144* during the infusion of 50 per cent glucose with and without fortification, and the nitrogen balance values during the administration of amigen and of gelatin P-20 are given in figure 2. The addition of methionine and tryptophane to the glucose infused resulted in diminished nitrogen excretion. Upon return to the infusion of glucose alone, the previous level of nitrogen output was restored. Nitrogen balances were compared during the amigen, gelatin P-20 and the respective protein-free periods. During the infusion of amigen, nitrogen excretion increased promptly, whereas with gelatin P-20 a delayed excretion occurred in both the precipitable gelatin and other urinary nitrogen. The plasma gelatin concentration was 1.6 grams per cent on the sixth day of infusion. It had decreased to 0.8 grams per cent by the third day of the subsequent protein-free glucose period.

In *dog 44* (fig. 3), comparisons were also made of the nitrogen excretion during infusions of gelatin P-20 and glucose, each with and without methionine and tryptophane supplementation. The urinary nitrogen and nitrogen balance values in figure 3 are represented by 4-day averages, except for the initial 2 days during and immediately following gelatin and the supplemented glucose infusions. Increased nitrogen output was observed during the administration of the gelatin solutions and was particularly marked with the injection of the 2x and 3x 'standard' gelatin concentrations. Again, the precipitable urinary gelatin (6) was approximately 50 per cent of that injected in each of the three periods.

A decreased excretion of undetermined nitrogen, from 0.54 to 0.33 gm. N per day, was noted with the first gelatin infusion but a gradual increase occurred during the later ones. These changes were small but consistent and seemed to vary with the state of protein depletion of the animal. Positive water balance occurred during the periods of gelatin infusion. These varied in degree with the concentration of

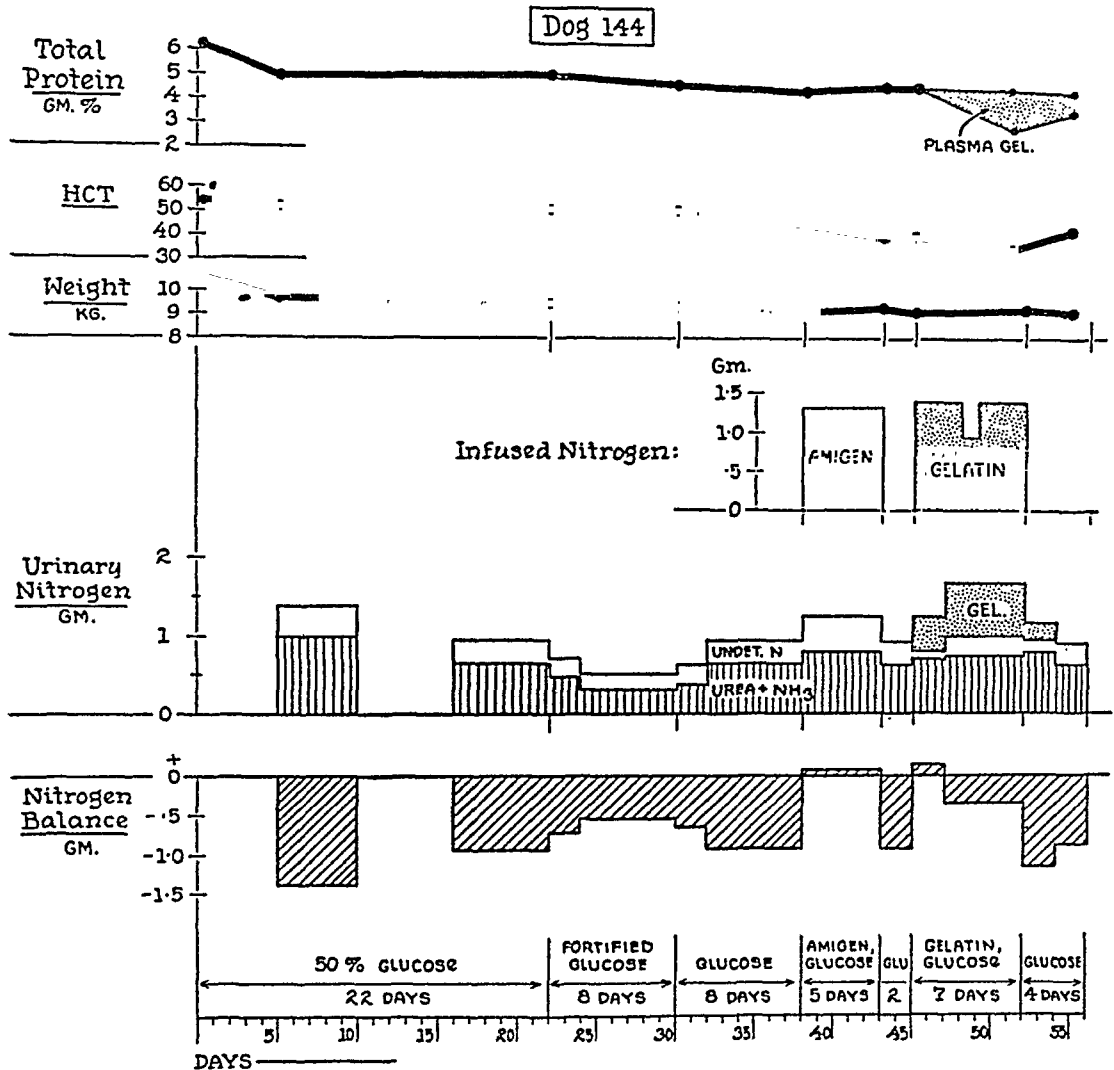


Fig. 2. NITROGEN BALANCES during infusions of glucose, glucose fortified with methionine and tryptophane, amigen and gelatin P-20. (Incomplete records for days 11 to 16 were due to difficulties in the infusion outfit.)

gelatin infused. They were followed by transient peaks in the excretion of urinary nitrogen (days 18-21, 115-121) which were mainly associated with a return of a diminished urine volume to control levels.

A comparison was made of the differences in nitrogen excretion during each of the three gelatin infusions, with that during their respective protein-free glucose periods. Thus the periods of gelatin supplemented with methionine and tryptophane were considered in relationship to their respective protein-free periods of glucose with methionine and tryptophane. Likewise, the infusion of gelatin alone was com-

pared with its control protein-free period of glucose alone. The nitrogen balance values of these three gelatin infusions were similar.

A gradual rise in nitrogen output, mainly urea and ammonia nitrogen, occurred during the 6-day period following the removal of methionine and tryptophane from the 50 per cent glucose which had been infused for 75 days. This is in contrast to a more rapid rise during a 2-day period in *dog 144* (fig. 2), wherein glucose with methionine and tryptophane had been given for only 8 days. A favorable nitrogen balance was associated with the injection of one-half the 'standard' amount of amigen.

The infusions were discontinued in this dog after completion of the 141st day, consequently the urinary nitrogen output other than that of precipitable gelatin was not followed.

The concentrations of plasma gelatin were plotted as additions to those of the native plasma proteins giving the values for total plasma colloid. The most rapid rise in plasma gelatin concentrations occurred during the first 4 days of gelatin infusion and maximal concentrations were usually reached by the 8th day. Average maximal concentrations were 1.5 and 2.95 gm. per cent with the administration of 1x and 3x standard gelatin solutions. As colloidal gelatin accumulated the native plasma protein levels were depressed and simultaneously evidence of hemodilution appeared in both the hematocrit and total plasma colloid values. The concentration of native plasma protein fell to 1.0 gm. per cent when the maximal gelatin concentration was 2.95 gm. per cent. Upon cessation of these injections, the plasma gelatin gradually disappeared within the following week, with a concomitant rise in the concentrations of native plasma proteins. Gelatin did not appear in the urine after the 4th day, when the plasma level had fallen to 0.5 to 0.7 gm. per cent.

Hyperglycemia and glycosuria did not appear in any of the dogs in this study. A gradual increase in urine volume up to 2x to 4x was noted in most dogs which were on experiment one month or more, but urinary nitrogen excretion was unaltered.<sup>7</sup>

In order to compare nitrogen utilization in the same and different dogs with varying degrees of protein depletion, the principles of Allison *et al.* (6) were employed. In the relationship of nitrogen balance and infused nitrogen, a comparison was made of the differences in nitrogen excretion during the protein and the average of the respective protein-free, glucose periods. The calculations were expressed as percentage of infused nitrogen retained during the period of protein infusion. The average nitrogen excretion during gelatin infusion, excluding the initial 2 days of diminished nitrogen output, was used in the computations of macromolecular gelatin utilization. The average excretion of urine nitrogen in the basal protein-free periods preceding and following the gelatin infusion was estimated from 4-day periods of relatively constant nitrogen output. In the after period it frequently took 4 to 10 days for an apparently steady state of nitrogen excretion to be attained. Rarely did the *pre* and *post* values coincide, the second figure being generally lower. The figures for 'retained nitrogen' are therefore presented as approximations (table 2).

<sup>7</sup> This occurred as readily on glucose alone, as it did when N- containing solutions were being injected. In the latter instances there were periods when rather marked variations in water balance occurred. These changes were quite abrupt and transient and were not associated with any observed clinical changes in the status of the animal.

The average values for 'retained nitrogen' in all experiments where amigen was infused was 76 per cent. When amigen-gelatin was infused 54 per cent of the nitrogen was retained. Gelatin, supplemented with methionine and tryptophane, in two experiments gave a 59.5 per cent retention when calculated from base-line periods of glucose alone. An average gelatin nitrogen retention of 23 per cent was observed in 6 experiments, where the base-period infusions were glucose alone. In 4 experiments both the gelatin and the glucose of the protein-free base periods were supplemented with methionine and tryptophane. These gave an average nitrogen retention of 28 per

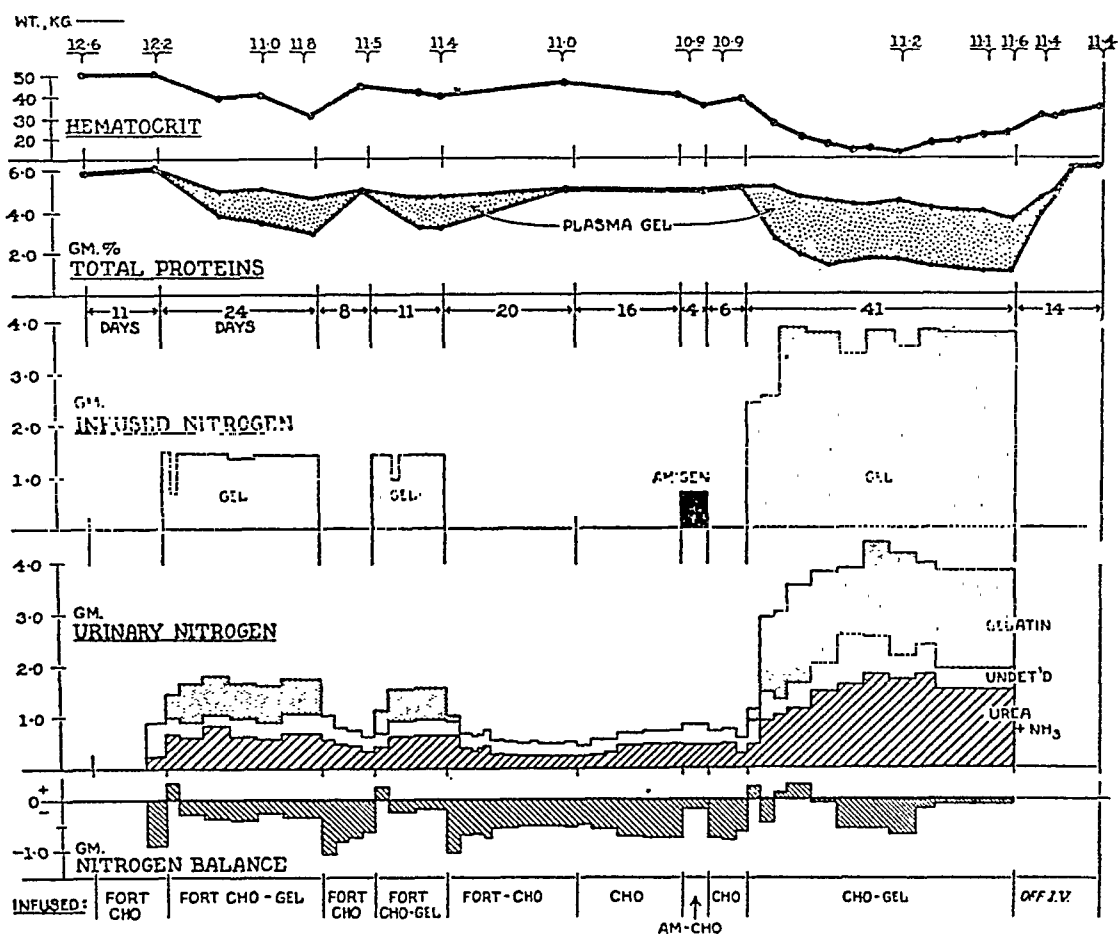


Fig. 3 NITROGEN BALANCES during the administration of fortified gelatin,  $\frac{1}{2}$  'standard' amigen and 2x-3x 'standard' gelatin compared with the respective amino acid, supplemented and non-supplemented glucose periods (dog 44).

cent. The individual values for the utilization of gelatin nitrogen varied considerably more than those for amigen.

In one experiment a comparison was made of the utilization of gelatin nitrogen given by vein, with continuous intragastric feeding. When given by vein 38 per cent of the gelatin nitrogen was retained, while only 17 per cent was retained when the gelatin was given into the stomach.

#### DISCUSSION

In a study of protein evaluation, adequate non-protein calories must be supplied to fulfill the gross energy needs of the organism tested. Calories were supplied in these

experiments by the continuous administration of 50 per cent glucose at a rate of 40 to 50 cal/kg/day (approximately 12 gm/m<sup>2</sup>/hr.), which according to Astwood (7) is the maximal amount of glucose which can be tolerated by dogs for 35 days or longer.

The excretion of urinary nitrogen during the initial period of equilibrium (6-13 days) was comparable to the data reported by Allison *et al.* (8) with oral protein-free diets of 70 cal/kg/day or more. The addition of methionine and tryptophane to the glucose resulted in a considerable diminution in nitrogen excretion during its administration, predominantly in the urea and ammonia nitrogen fractions. These observations confirm those of Allison in the dog (6) and Brush in the rat (9) in that methionine appears to spare nitrogen catabolism in these animals.

In the single experiment comparing the different routes of administration no alteration in nitrogen excretion was noted when glucose was injected continuously into the vein or stomach in successive periods.

The amount of infused nitrogen, 120 mg/kg/day, was selected in order to maintain a zero or slightly negative nitrogen balance, a range in which nitrogen is effectively utilized.

The prompt, sustained elevation in nitrogen excretion with amigen, above that during the protein-free base line was composed of approximately equal amounts of both the urea-ammonia and the undetermined nitrogen fractions. The approximate average total value of this elevation was 20 per cent when calculated as percentage of infused nitrogen. The marked rise in nitrogen excretion with gelatin infusion consisted mainly of the precipitable gelatin and the urea-ammonia fractions, approximately 50 per cent and 20 per cent respectively, whereas the maximum increase in the undetermined nitrogen was 7 per cent. The gradual changes in nitrogen output and plasma colloid concentration during and after gelatin injections were probably associated with changes in water balance; fluid, colloid and electrolyte shifts; and a slower, more gradual metabolism of macromolecular gelatin.

Plasma gelatin concentration increased to levels of 2.95 gm. per cent with the 3x 'standard' infusion, whereas that of the native plasma protein was simultaneously depressed to 1.0 gm. per cent. The total plasma colloid concentrations at this time were below those of the control period, reflecting hemodilution. With the injection of smaller amounts of gelatin the plasma levels were proportionately less and the total colloid concentrations were relatively unchanged. Such minor changes in the levels of total colloid, and large shifts in the component fractions probably indicated an oncotic mechanism regulating the concentrations of plasma proteins, or perhaps a depression of native plasma protein synthesis during periods of gelatin infusion.

Investigators have computed absorbed nitrogen to be ingested nitrogen minus fecal nitrogen in studies of the evaluation of proteins fed orally. Similarly here an analogy might be made in which 'absorbed nitrogen' would be equal to infused nitrogen minus urine gelatin nitrogen and the extra amino acid nitrogen in both free and combined forms. The amount of infused gelatin which was not excreted as precipitable gelatin and extra amino nitrogen would be available for metabolism. By this method of calculation the values for 'retained nitrogen' during gelatin infusions would be increased approximately twofold. This, however, would be obviated when net protein-nitrogen gain to the animal per unit protein-nitrogen intake was considered.

In most dogs, by various methods of calculation, there was evidence that a quantity of nitrogen calculated as equivalent to 10 to 30 per cent of the infused gelatin nitrogen did not appear in the urine as precipitable gelatin or as increased urea-

TABLE 2. RETAINED NITROGEN

DOG	EXPER., DAYS	AMIGEN	AM.-GEL.	GEL.-P-20	GEL.-P-20
			% Infused (a)		gm/day (b)
239	10-15	79	53	65 F <sup>1</sup>	
	16-21				
	28-35				
44	13-20	80	59		
	21-28				
907	10-15	72	50	54 F <sup>1</sup>	
	16-21				
	28-37				
1045	14-18	74			
144	38-43	77		35	.52
	46-52				
189	27-48			4	.07
1039	13-25			31 F	.47
181	11-16	68		22 F	.37
	34-38			32	.57
	74-77				
100	22-29			38	.53
	44-51			17 Fst.	.24
144	14-26			19	.68
44	12-35	82		32 F	.44
	44-54			27 F	.36
	90-94				
	100-141			11	.41

(a) Calculated as % nitrogen infused during administration of amigen, amigen-gelatin P-20, and gelatin P-20 with and without methionine and tryptophane. (b) Calculated as grams of infused gelatin nitrogen retained per day.

F<sup>1</sup> = Supplemented Gel-P-20 compared with non-supplemented glucose protein-free period. F = Gel-P-20 supplemented with methionine and tryptophane. Fst. = Gel-P-20 injected by intragastric catheter.

ammonia or undetermined nitrogen either during the gelatin infusion or in the following protein-free glucose period. In the calculation of the percentage retained nitrogen, the average excretion of urine nitrogen in the basal-protein-free periods preceding and following the gelatin infusion was estimated from 4-day periods of relatively constant

nitrogen output. In the after period it frequently took 4 to 10 days for an apparently steady state of nitrogen excretion to be attained. Rarely did the *pre* and *post* values coincide, the second figure being lower. This prolonged period required for a constant nitrogen output to be obtained was in part due to the excretion of gelatin present in the body at the end of gelatin infusion. Precipitable gelatin in detectable amounts was excreted until the plasma level reached approximately 0.5 to 0.7 gm. per cent gelatin. The amount of precipitable gelatin excreted in the after protein-free glucose period was 1 to 4 per cent of the total gelatin infused. The excretion of extra urea and ammonia nitrogen in this period was calculated to be up to 10 per cent of the infused gelatin nitrogen. This extra urea and ammonia nitrogen was excreted during the initial 4 to 10 days of the after-glucose period and prior to the establishment of the steady state of nitrogen excretion. The nature of the 'stored' products, whether as gelatin nitrogen itself or partial split products, cannot be answered by these data. However, in two dogs receiving gelatin, the quantity of extra nitrogen excreted in the initial 'carry-over' phase of the following protein-free period was comparable in magnitude to the amount calculated as 'retained nitrogen' during the period of gelatin infusion. This extra quantity of nitrogen was composed mainly of urea-ammonia nitrogen and to a lesser extent of undetermined and precipitable gelatin nitrogen. The values for 'retained nitrogen' calculated during the gelatin infusions were low in these dogs (189 and 144, *exper. 2*).

Histologic examination of the tissues of the two protein-depleted animals (*dogs 44 and 144*), killed after long, chronic experiments, revealed changes similar to the observations reported by Parkins *et al.* (10). No irreversible lesions in the kidney and liver that could be attributable to the infused gelatin were noted.<sup>8</sup>

Weight loss after the initial 1- to 2-week period of equilibrium was relatively small in all dogs, and upon completion of the experiments these dogs appeared normally active and in good physical condition.

#### SUMMARY

Dogs receiving 45 to 50 cal/kg/day supplied by 50 per cent glucose infused continuously have, in general, urinary nitrogen excretions of 100 to 110 mg/kg/day during an initial 6- to 13-day period. These nitrogen excretions are comparable to those reported by others feeding protein-free diets by mouth containing 70 to 80 cal/kg/day.

The diminished excretion of urinary nitrogen during infusions of 50 per cent glucose supplemented with methionine and tryptophane confirm previous reports. The calculated nitrogen retention values for amigen in these studies are similar to, or somewhat higher than, those reported for single daily injections. The average of these values is 76 per cent.

The average nitrogen retention calculated for gelatin P-20 is approximately 27 per cent. Considerable variation in the individual values was noted. No significant difference in these values was observed for gelatin P-20, with or without a methionine tryptophane supplement, when compared to the respective protein-free base line

<sup>8</sup> We wish to thank Dr. Herbert Ratcliffe, Dept. of Pathology, University of Pennsylvania School of Medicine and Chief Pathologist of Philadelphia Zoological Gardens, for these examinations.

periods. The substantial increases in excretions of urea and ammonia nitrogen during infusions of gelatin P-20 are interpreted as an indication of the metabolism of the gelatin not excreted as such in the urine.

The authors express their thanks to Dr. D. Tourtellotte who throughout these experiments prepared all solutions used for intravenous feeding, except for the amigen solutions.

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# DISCHARGE OF ADRENOCORTICOTROPHIC HORMONE FROM TRANSPLANTED PITUITARY TISSUE<sup>1</sup>

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**O**BSERVATIONS on the adrenocorticotrophic activity of pituitary transplants have been incidental to studies on the mechanism of regulation of pituitary gonadotrophic function. Hill and Gardner (1) have described normal adrenals in two hypophysectomized mice with intratesticular transplants of pituitary tissue. In guinea pigs with intraocular transplants of pituitary tissue Schweizer, Charipper and Haterius (2) state, "The adrenals especially were well differentiated both in the cortical and medullary regions." However, Schweizer, Charipper and Kleinberg (3) later reported figures which show a reduction in the weights of the adrenals of similarly prepared animals. Cutuly (4) demonstrated that intrasellar, but not intraocular, grafts of pituitary tissue will maintain the weight of the adrenals of hypophysectomized rats. It would therefore appear that under certain circumstances pituitary transplants secrete adrenocorticotrophic hormone (ACTH).

Stalk-section experiments have shown that neural connections between the hypothalamus and the pituitary are essential neither for the pituitary-induced adrenal hypertrophy which accompanies chronic stress (5, 6) nor for the prompt discharge of ACTH during acute stress (7). However, Harris (8) has called attention to the possible importance of an hypophyseal portal venous system in the regulation of pituitary activity; according to Harris, this vascular link may regenerate after stalk section. Therefore, it was thought important to study the ability of pituitary tissue, implanted at sites removed from the hypothalamus, to discharge ACTH in response to acute stress.

## METHODS

Male rats from the Sprague-Dawley farm were divided into 3 groups, as follows: the animals of *group 1* remained as controls; those of *group 2* were completely hypophysectomized; and the rats of *group 3* were first completely hypophysectomized and then implanted with pituitary tissue at one or more of 3 sites (sella turcica, anterior chamber of the eye, spleen).

Hypophysectomy was performed by the parapharyngeal approach. The technic employed for transplantation was the following: The pituitary of a rat was sucked

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<sup>2</sup> Fellow of the American Bureau for Medical Aid to China.

out of the sella turcica into a bottle containing a solution of 0.9 per cent sodium chloride at 37° C. The anterior pituitary tissue was cut into small pieces and a fragment, amounting to one-quarter or less of the gland, introduced into the anterior chamber of the eye of the same rat (auto-transplant), through a slit at the corneal-scleral junction, or into the anterior chamber of the eye of a recipient rat (homo-transplant) which had been hypophysectomized a few minutes earlier. In other rats, the tissue was auto- or homo-transplanted into the spleen. In still other animals, the gland was sucked out of the sella turcica and immediately returned to the pituitary fossa. In a few animals, transplants were introduced into the anterior chamber of the eye and a number of days later a homo-transplant of anterior pituitary tissue was introduced into the spleen. Serial sections of the organs receiving transplants were stained with hematoxylin and eosin, or by a Mallory-azan technic as modified by Koneff (9). The following measurements were employed to evaluate the functional activity of the transplanted tissue: body, adrenal, and testicular weights, and the rate of release of ACTH from the graft in response to a sudden stress.

It has been demonstrated (10) that discharge of ACTH from the pituitary is followed by an immediate reduction in adrenal ascorbic acid. In the present study, depletion of adrenal ascorbic acid in response to histamine stress was employed as a method for determining the ability of transplants to discharge ACTH. From 13 to 54 days after implantation of pituitary tissue the rats were anesthetized with sodium pentobarbital and the left adrenal was removed and prepared for an analysis of the control level of ascorbic acid; histamine (1.0 mg/100 gm. body weight) was then injected intravenously and one hour later the right adrenal was removed and prepared for the analysis of ascorbic acid. The quantity of ACTH released from the transplant in response to histamine is proportional to the degree of depletion of adrenal ascorbic acid, as measured by the difference between the concentration in the left and right adrenal glands. The details of the method have been presented in a previous paper (7).

In the experiments involving transplants to the eye or spleen the sellar region was examined for the presence of residual anterior pituitary tissue. Direct inspection of the sella under 2 times magnification revealed no residual pituitary tissue in any instance. Furthermore, hypophyseal tissue was not seen in the serially cut sections of the entire region of the sella turcica (stained with hematoxylin and eosin) of 3 of the animals with extrasellar grafts (last 3 animals in table 1).

#### RESULTS

Of 36 hypophysectomized rats transplanted with pituitary tissue only 13 showed histological evidence of the presence of viable glandular cells in the grafts. One rat had viable grafts in both the anterior chamber and the spleen. The data for these 13 animals and for the intact and hypophysectomized control rats are presented in table 1.

*Histology of the Transplants.* In general, transplantation to the anterior chamber of the eye was more successful than transplantation to the spleen. The area of implant in the 23 rats with non-functional grafts was made up entirely of connective tissue stroma and cellular debris with no viable glandular cells present. A number

of the successful transplants consisted of layers of glandular cells arranged in an arc which partially enclosed a mass of connective tissue stroma and cellular debris. A few of the grafts consisted of a spherical clump of cells (see fig. 1, *left*). The viable areas of the transplants had a moderate number of capillaries. Practically all of the glandular cells of the viable transplants were chromophobic; in a few grafts the cytoplasm of a small number of cells were weakly basophilic or acidophilic.

*Functional Activity of the Transplants.* The intraocular and intrasplenic transplants were incapable of supporting growth. The control rats grew normally throughout the experimental period. The completely hypophysectomized rats barely maintained their initial body weights with the exception of one which gained weight; in this rat, the pituitary was removed at an early age. In the viable transplant group, 7 rats maintained their original weight. Six rats gained appreciably in weight; but in 5 of these, which were operated upon at a young age, the weight gain was no greater than that which occurs in non-transplant animals hypophysectomized at a similar age. In only one rat was the gain in weight (from 92 to 160 gm.) of sufficient magnitude to suggest that it resulted from elaboration of growth hormone by the pituitary transplant.

Testicular weights were determined in only 2 of the 13 transplant rats. Both of these animals had atrophic gonads, a fact which indicates the inadequacy of the pituitary transplants in regard to gonadotrophic hormone activity.

With the exception of one animal with a sellar graft, the rats with pituitary transplants were found to have atrophic adrenals (table 1). The decrease in weight of the adrenals was in most instances as great as that which occurred in the hypophysectomized animals without pituitary grafts. Following hypophysectomy the concentration of adrenal ascorbic acid was markedly reduced in the animals not having transplants. The level of adrenal ascorbic acid of the rats with pituitary transplants was intermediate (256 to 452 mg/100 gm.) between that of the intact control rats (383 to 430 mg/100 gm.) and that of the hypophysectomized rats without transplants (149 to 271 mg/100 gm.). These data suggest that the functional activity of the adrenal cortex of hypophysectomized rats with transplants was greater than that of hypophysectomized rats without transplants.

One hour after the injection of histamine there was a marked reduction in the concentration of adrenal ascorbic acid in the control rats (table 1). The concentration of ascorbic acid in the left adrenal, removed before histamine injection, was about twice that in the right adrenal removed one hour after histamine. Neither the hypophysectomized animals without transplants nor those with non-viable grafts responded to histamine with a significant drop in adrenal ascorbic acid. The results for the hypophysectomized animals without transplants are presented in table 1. The average of the differences in concentration of ascorbic acid between the left and right glands (left-right) of the animals with non-viable grafts was  $-10$  mg/100 gm. (range  $+22$  to  $-41$ ), i.e., the average concentration of ascorbic acid in the right adrenals removed after histamine was greater than that of the left adrenals removed before histamine. Of the 13 animals with evidence of viable transplant tissue, 11 responded to histamine with significant decreases in adrenal ascorbic acid. The degree

of reduction in adrenal ascorbic acid was variable in this group and in only a few instances did the degree of depletion approach that seen in the normal animals. In

TABLE 1. DISCHARGE OF ACTH FROM ADENOHYPHYPHYSEAL TRANSPLANTS IN RESPONSE TO HISTAMINE STRESS

GROUP	BODY WEIGHT		ADRE- NAL WEIGHT	TESTES WEIGHT	ADRENAL ASCORBIC ACID			SITE OF TRANS- PLANT	HOMO OR AUTO
	initial	final			left	right	differ- ence left- right		
	<i>gm.</i>		<i>mg.</i>	<i>gm.</i>	<i>mg/100 gm. tissue</i>				
Control	75	270 (46) <sup>1</sup>	28.3		383	225	158		
	60	264 (46)	24.4		426	225	201		
	62	276 (46)	34.8		430	231	199		
	192	250 (13)	27.6	2.77	402	194	208		
	194	240 (13)	27.6	2.71	408	248	160		
	230	298 (14)	32.0	2.60	405	206	199		
	200	264 (14)	29.9	2.90	422	204	218		
	190	270 (15)	32.5	3.00	388	183	205		
	170	210 (15)	26.9	2.16	425	175	250		
Hypophysecto- mized	53	72 (51) <sup>2</sup>	6.4		183	204	-21		
	105	100 (51)	8.2		235	230	5		
	160	154 (13)	11.8	0.89	271	297	-26		
	150	140 (13)	17.7	0.22	235	200	35		
	172	170 (14)	12.8	0.70	268	269	-1		
	164	145 (14)	18.1	0.56	158	152	6		
	152	140 (15)	13.8	0.16	260	270	-10		
	156	140 (15)	15.4	0.71	149	147	2		
Transplant	92	160 (21) <sup>2</sup>	35.0		377	230	147	Sella	Auto
	150	160 (35)	14.4		332	233	99	Sella	Homo
	136	140 (36)	15.7		256	181	75	Spleen	Homo
	128	130 (27)	14.8		337	127	210	Chamber	Homo
	124	130 (40)	12.8		435	364	71	Sella	Homo
	48	82 (54)	6.1		372	294	78	Chamber	Homo
	58	80 (54)	5.9		263	238	25	Chamber	Auto
	50	84 (54)	7.9		317	276	41	Spleen	Auto
	200	200 (13)	22.0		315	230	85	Chamber	Auto
	52	92 (54)	8.4		345	207	138	Chamber	Homo
	171	171 (13)	14.2		289	235	51	Chamber	Auto
	194	190 (13)	15.1	1.34	302	239	63	Chamber	Auto
	51	70 (32)	13.6	0.14	452	329	123	Chamber + Spleen	Auto Homo

<sup>1</sup> Figures in parentheses denote number of days elapsed between measurements of initial and final body weights.

<sup>2</sup> Figures in parentheses denote number of days elapsed between operation or implantation and autopsy.

two of the 13 animals the decreases in adrenal ascorbic acid (25 and 41 mg/100 gm.) cannot be considered significant.

## DISCUSSION

Harris (8) has presented three criteria for the adequate study of pituitary transplants. He states, "First, the grafted tissue should be situated at a distance from the normal site of the gland thus obviating the danger of vascular or nervous repair; second, the studies should be extended over a period sufficient to ensure that any effects observed are due to functional activity of the transplant, and not to absorption of an implant; and third, serial sections through both the transplant and sella turcica with surrounding tissue are necessary to control the activity of the graft and the complete removal of the pituitary." In the present study all 3 criteria have been fulfilled in 3 of the animals with transplants. Complete serial section of the sellar region in these 3 rats failed to reveal adenohypophyseal tissue. In the other animals with viable transplants, the sellar region was examined only grossly and not microscopically; no residual pituitary tissue was observed. Despite the lack of serial sections in these rats, it appears most unlikely that the reduction of adrenal ascorbic acid was due to the release of ACTH from residual tissue in the sella turcica, because all hypophysectomized animals without transplants and all those with non-viable transplants failed to show a significant reduction in adrenal ascorbic acid in response to acute stress. Therefore, it would seem to be reasonably certain that the depletion of adrenal ascorbic acid in the animals with viable transplants was due to the discharge of ACTH from such transplants.

In every animal the period of time elapsed between the transplantation and the testing of functional activity of the graft was sufficiently long to eliminate simple absorption of the ACTH originally present in the transplant as a factor influencing the results of this study. A single injection of ACTH in doses much larger than the amount stored in the rat pituitary has an action which lasts but a few hours (10). It is extremely unlikely that the transplant acted as an inert depot of ACTH which released the trophic at an accelerated rate in response to stress. Furthermore, non-viable grafts showed no evidence of adrenocorticotrophic activity.

The weights of the adrenals of the animals with transplants were about equal to those of the adrenals of hypophysectomized rats without transplants. However, the pre-stress adrenal ascorbic acid values suggest that the viable grafts exerted some trophic action on the adrenals during the period between implantation of pituitary tissue and injection of histamine. The lack of maintenance of adrenal weight in the animals with transplants could conceivably be explained on the basis of an inadequacy of adrenocorticotrophic function of the graft. However, the authors are not inclined to favor this explanation because the graft had a store of ACTH which could be rapidly released in response to an acute stress. A more likely explanation for the reduction in adrenal weight is that it represents a partial atrophy associated with a decrease in the requirement for cortical steroids in animals with lowered metabolic rates resulting from thyroid atrophy. Unfortunately thyroid function was not examined in our animals, but Cutuly (4) has demonstrated that thyroid atrophy does occur in rats with intraocular transplants of pituitary tissue. Evidence that a reduction in metabolic activity decreases the requirement for adrenal cortical hormones has been presented by Sayers and Sayers (10).

In response to equal doses of histamine, a greater quantity of ACTH was dis-

charged from the intact pituitary than from the pituitary graft, as measured by the adrenal ascorbic acid-depletion method. The most reasonable explanation for this quantitative difference in ACTH discharge is that the amount of viable tissue in the transplant was in all instances less than one-twentieth the normal mass of the anterior pituitary. Furthermore, the rate of exit of ACTH from the graft may be limited by the nature of its blood supply. The possibility cannot be ruled out, however, that removal of the pituitary from its normal site may have resulted in the loss of regulatory influences from the hypothalamus. Also, a reduction in sensitivity of the target gland to ACTH must be considered as a factor influencing the decreased response of the adrenal to stress. The metabolic activity of the adrenal cortex, like that of other tissues of the organism, may have been reduced. Even if the same quantity of ACTH had been discharged from the transplanted pituitary as from the intact pituitary, it might have exerted less influence because of the decreased responsiveness of the adrenal gland.



Fig. 1. *Left*: CROSS SECTION OF EYE OF RAT with viable transplant. The cornea and anterior chamber are on the right. The dark-staining, dome-shaped mass projecting into the anterior chamber is the pituitary transplant. *Right*: Photomicrograph under high power magnification of a typical area of the viable pituitary transplant shown at the left.

The complexity of adenohypophyseal physiology precludes a definite answer to the question why the transplants were apparently less efficient than the normal pituitary. However, the data conclusively demonstrate that neural connections or a portal venous system between the hypothalamus and the pituitary are not necessary for the prompt discharge of ACTH in response to stress. Evidence that a humoral mechanism regulates pituitary adrenocorticotrophic activity has been presented by Sayers and Sayers (11) who demonstrated that pretreatment of rats with adrenal cortical hormone prevents the discharge of ACTH which normally follows stress. The experimental results have been interpreted to mean that the rate of discharge of ACTH from the pituitary is inversely proportional to the level of adrenal cortical steroids in the body fluids. Stress initiates a release of ACTH by increasing the rate of utilization of adrenal cortical hormone in the organism. This release occurs from auto- and homo-transplants without neural or portal venous connections to the hypothalamus as well as from the normal pituitary *in situ*.

Since this manuscript was prepared for publication Dr. Claude Fortier has in-

formed the authors that he has obtained results in conformity with those presented above.

#### SUMMARY

Intraocular and intrasplenic grafts of adenohypophyseal tissue in hypophysectomized rats promptly discharge adrenocorticotrophic hormone (ACTH) in response to histamine stress. The amount of ACTH released is less than that released by the adenohypophyses of intact control rats given the same dose of histamine; this difference is most likely accounted for by the small quantity of surviving pituitary tissue in the transplants. Other possible explanations are presented. The results have been interpreted to mean that neural or vascular connections between the hypothalamus and the anterior pituitary are not essential for the immediate discharge of ACTH in acute stress.

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# ADRENOCORTICOTROPHIC EFFECT OF STRESS AFTER SEVERANCE OF THE HYPOTHALAMO-HYPOPHYSEAL PATHWAYS

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THE various morphological (1-5) and chemical (6-11) alterations of the adrenal cortex, which are observed to follow exposure to stress, constitute adequate evidence of an increased release of pituitary adrenocorticotrophic hormone. Still more direct proof of this process has recently been furnished (12, 13) through the demonstration of a marked depletion in the corticotrophin content of the pituitary, as judged by bioassay, in animals which had been exposed to noxious stimuli. Regarding the mechanism underlying this stress-induced liberation of corticotrophin, there is disagreement between the proponents of a direct humoral (8-14) and of an indirect neural or neurohumoral (15, 16) theory of mediation.

In relation to the latter possibility, the few published reports of the adrenal response to stress after severance of the hypothalamo-hypophyseal pathways, have yielded somewhat conflicting and inconclusive results. This is partly due to the technical difficulty inherent in transplanting the pituitary or transecting its stalk, and partly to the lack of a sensitive and reliable index of corticotrophic function. Such an index is now available, and we have taken advantage of the Sayers adrenal ascorbic acid test (17) to further elucidate this problem.

## EXPERIMENT A. ADRENAL RESPONSE TO STRESS AFTER PARTIAL HYPOPHYSECTOMY

*Materials and Methods.* Twenty-four male piebald rats, averaging 150 gm. in weight, were divided into three groups. The animals of *Group I* served as controls, those of *Group II* were hypophysectomized, by the parapharyngeal approach, while in *Group III*, a partial hypophysectomy was performed. In the latter operation, the rostral part of the anterior lobe and the whole posterior lobe were aspirated through a very fine cannula, thus severing all connections of the remaining part of the gland with the floor of the 3rd ventricle. Forty-eight hours after the operation, the animals of all three groups were submitted to unilateral (left) adrenalectomy under light ether anesthesia, exposed to cold (0°C) for one hour, and then killed by bleeding. The right adrenal was removed immediately thereafter and both glands were dissected free of adherent fat and connective tissue, weighed and ground in 8 cc. of 4 per cent trichloroacetic acid for ascorbic acid determination (method of Roe and Kuether, 18). The completeness of the hypophysectomy in *Group II*, as well as the state of the hypophyseal remnant in *Group III*, was checked through careful examination of the sellar region with a 5 X magnifying glass.

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*Results.* Table 1 compares the ascorbic acid concentration of the left (removed before exposure to stress) and right (removed after killing) adrenals, the difference in concentration being considered as a quantitative index of corticotrophic stimulation. In the control animals (*Group I*), a marked decrease in the adrenal ascorbic acid concentration is observed as a combined effect of the surgical intervention (adrenalectomy) and exposure to cold. No such decrease is evidenced in the completely hypophysectomized animals (*Group II*). On the other hand, partial hypophysectomy, with the associated severance of the hypothalamo-hypophyseal pathways, did not prevent the occurrence of a stress-induced ascorbic acid discharge (*Group III*). Indeed, the latter did not differ significantly from that observed in the controls. It is of interest that the ascorbic acid concentration of the left adrenal is markedly higher, both in the completely and in the partially hypophysectomized animals than in normal controls.

TABLE 1. ADRENAL ASCORBIC ACID CONCENTRATION BEFORE AND AFTER SURGICAL TRAUMA AND EXPOSURE TO COLD IN NORMAL, PARTIALLY HYPOPHYSECTOMIZED AND COMPLETELY HYPOPHYSECTOMIZED ANIMALS

GROUP	NUMBER OF ANIMALS	TREATMENT	ASCORBIC ACID LEFT ADRENAL <sup>1</sup> (mg/100 mg adrenal tissue)	ASCORBIC ACID RIGHT ADRENAL <sup>2</sup> (mg/100 mg adrenal tissue)	D <sup>3</sup>	D <sup>4</sup>
I	10	None	0.449 ± 0.018	0.353 ± 0.014	-0.106 ± 0.025	22.2 ± 5.1
II	6	Complete hypophysectomy	0.536 ± 0.014	0.561 ± 0.053	+0.025 ± 0.050	5.5 ± 11.2
III	8	Partial hypophysectomy	0.541 ± 0.029	0.445 ± 0.021	-0.105 ± 0.031	17.8 ± 5.3

<sup>1</sup> Removed prior to surgical trauma and exposure to cold. <sup>2</sup> Removed after killing. <sup>3</sup> Difference between pre-stress (*left*) and post-stress (*right*) adrenal ascorbic acid levels. <sup>4</sup> Calculated by the formula: 
$$\frac{(\text{L. adren. asc. ac. conc.} - \text{R. adren. asc. ac. conc.}) \times 100}{\text{L. Adren. asc. ac. conc.}}$$

This is in agreement with the observation of an initial rise in the adrenal ascorbic acid level after hypophysectomy (19).

#### EXPERIMENT B. ADRENAL RESPONSE TO STRESS AFTER PITUITARY TRANSPLANTATION IN THE HYPOPHYSECTOMIZED ANIMAL

*Materials and Methods.* Twenty-four male piebald rats of an average initial weight of 140 gm. were used in this experiment. Six animals served as controls (*Group I*). In the others the pituitary was transplanted by the following technique: The whole anterior lobe of the pituitary of a freshly killed animal of the same sex and weight was carefully aspirated into the bore of a no. 18 spinal puncture needle. The needle was inserted into the anterior chamber of the eye through a meridional slit previously made in the cornea, care being taken to prevent herniation of the iris through the slit. The gland was then deposited by pushing in the plunger of the needle. The animals were hypophysectomized 24 hours later. At the end of 4 weeks, they were submitted (as in the first experiment) to a left adrenalectomy, followed by exposure to cold (0° C.) during one hour, after which they were killed. Ascorbic acid

was determined in both adrenals in the manner previously described. The graft-containing eye was removed, its posterior half cut away and the remaining portion fixed in Zenker-formol. The pituitaries of the normal controls and the sellar regions of the hypophysectomized animals were similarly fixed, the latter prior to decalcification. The testes and seminal vesicles were fixed in Bouin's fluid. Zenker-fixed material was sectioned at  $7\ \mu$  and stained with Mallory's triple stain or with Masson's modified trichrome. Other tissues were cut at  $5\ \mu$  and stained with H and E. Completeness of the hypophysectomy was ascertained by microscopic examination of serial sections of the sellar region.

TABLE 2. ADRENAL ASCORBIC ACID CONCENTRATION BEFORE AND AFTER SURGICAL TRAUMA AND EXPOSURE TO COLD IN NORMAL ANIMALS AND IN HYPOPHYSECTOMIZED BEARERS OF OCULAR PITUITARY IMPLANT

GROUP	TREATMENT	ANIMALS	ASCORBIC ACID LEFT ADRENAL <sup>1</sup> (mg/100 mg. adrenal tissue)	ASCORBIC ACID RIGHT ADRENAL <sup>2</sup> (mg/100 mg. adrenal tissue)	D <sup>3</sup>	D <sup>4</sup>
I	None	Average of 6	0.334 $\pm$ 0.027	0.233 $\pm$ 0.011	-0.111 $\pm$ 0.028	32.2 $\pm$ 8.1
II	Hypophysec- tomy + pi- tuitary im- plantation	A	0.385	0.342	-0.043	11.1
		B	0.280	0.175	-0.105	37.5
		C	0.653	0.443	-0.210	32.2
		D	0.357	0.222	-0.135	37.8
		Average of 4	0.419 $\pm$ 0.081	0.296 $\pm$ 0.061	-0.123 $\pm$ 0.035	29.6 $\pm$ 8.3

<sup>1</sup>Removed prior to surgical trauma and exposure to cold. <sup>2</sup>Removed after killing. <sup>3</sup>Difference between left and right adrenal ascorbic acid levels. <sup>4</sup>Calculated by the formula: (L. adren. asc. ac. conc. - R. adren. asc. ac. conc.)  $\times$  100

L. adren. asc. ac. conc.

*Results.* Of the 18 experimental animals, five were lost before the termination of the experiment and nine were discarded, either because of the unsatisfactory state of the transplant, or because pituitary remnants were found in the course of the microscopic examination of the sella turcica. Four animals remained which met both requirements, i.e. adequate state of the transplant and ascertained completeness of hypophysectomy.

*Adrenal ascorbic acid.* The adrenal ascorbic acid concentrations in both groups, prior to and after exposure to stress, are listed in table 2. Here we denervated the pituitary through another technique and yet all the experimental animals again showed a stress-induced loss of adrenal ascorbic acid. The average discharge, representing 29.6 per cent of the initial (left adrenal) level, did not differ significantly from the corresponding value of 32.2 per cent observed in the controls.

*Weight changes.* A slight decrease in body weight was noted in the first days after hypophysectomy. This was followed by a gradual return to the initial level by

the end of the experimental period. None of the grafted animals evidenced any actual increase above the initial level. The organ weights (table 3) showed a wide range of variability. While 3 of the experimental animals presented a marked atrophy of the adrenals, testes and seminal vesicles, with a slighter decrease in thymus weight, a fourth one (*rat II C*), the bearer of a particularly well differentiated pituitary transplant, did not show any atrophy of the above-mentioned organs.

*Histology.* The ocular implants were found attached to the iris, close to the ciliary margin. One implant (*rat II A*) had been almost completely replaced by connective tissue. Interestingly, this corresponded to the lowest (11.05%) adrenal ascorbic acid discharge. The others were well maintained and showed typical nest formations surrounded by narrow capillary sinuses containing erythrocytes (figs. 1-3). Differential staining showed a great predominance of unusually large chromophobe elements, occasional clusters of sparsely granulated eosinophiles and rare hypochromophil cells, whose identity could not be ascertained. Well characterized baso-

TABLE 3. ORGAN WEIGHTS<sup>1</sup> 28 DAYS AFTER HYPOPHYSECTOMY AND PITUITARY TRANSPLANTATION

GROUP	TREATMENT	ANIMALS	ADRENALS	THYMUS	TESTES	SEMINAL VESICLES
I	None	Average of 6	13.9 ± 0.05	232 ± 9	1504 ± 85	180 ± 29
II	Hypophysectomy + pituitary implanta- tion	A	7.2	223	341	55
		B	6.2	216	342	76
		C	10.3	220	1727	194
		D	4.9	121	167	25
		Average of 4	7.1 ± 1.2	195 ± 25	644 ± 362	87 ± 37

Weights are expressed in mg/100 gm. of body weight.

phils could not be demonstrated. The testes and seminal vesicles of one animal (*rat II C*) were well maintained and presented a normal histological structure. The others showed various degrees of involution of the tubular and interstitial elements.

#### DISCUSSION

In regard to some of its trophic functions, the anterior pituitary may evidence a basic rhythm of secretory activity even after separation from the overlying nervous centers. This is probably controlled through purely hormonal means but nevertheless seems to require the integrity of nervous connections for finer adjustments to environmental changes (20). Such appears to be the case for its thyrotrophic (21-23) and gonadotrophic (16, 24-26) functions, but there is no conclusive evidence to prove a nervous regulation of corticotrophin secretion. Several authors (22, 27, 28) found that the adrenal cortex remains normal in weight and histological appearance after pituitary-stalk section in the rat. Moreover, both in the rat (22) and in the dog (29) this operation failed to prevent the adrenal cortical hypertrophy which normally follows upon exposure to cold, in particular (22, 29), or stress in general (29a). Differing accounts have been given regarding the effect upon the adrenal of hypophyseal transplantation after hypophysectomy. Hill and Gardner, 1936 (30), work-

ing with mice found no adrenal atrophy  $4\frac{1}{2}$  months after this intervention. Essentially similar findings have been obtained in the guinea-pig (31, 32) and rat (33). However, Cutuly, 1941 (34), experimenting with sellar and ocular transplants in the hypophysectomized rat noted that only sellar grafts were adrenotrophic, while

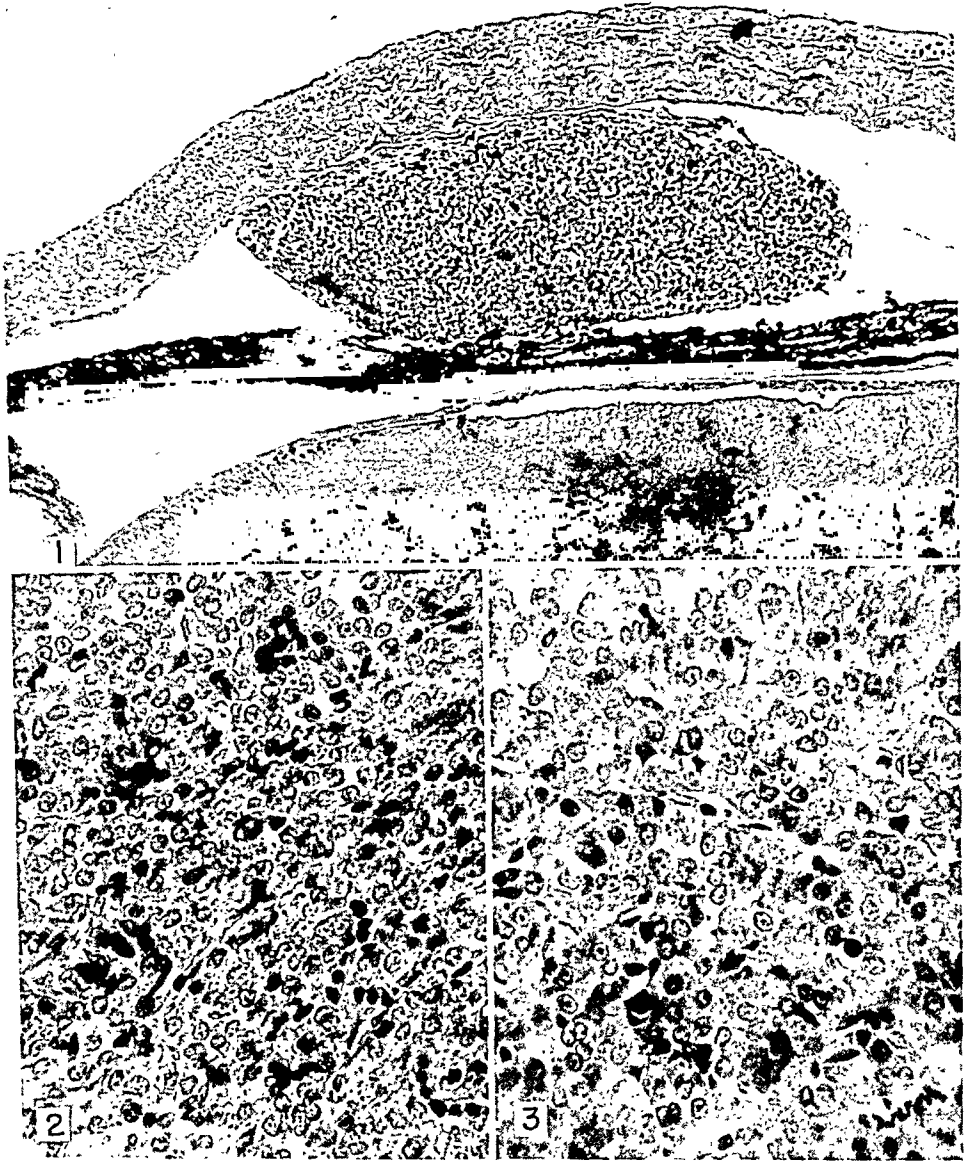


Fig. 1. LOW POWER PHOTOMICROGRAPH showing intraocular pituitary transplant and its attachment to iris (x 50).

Fig. 2. SAME PITUITARY TRANSPLANT under higher magnification. Note predominance of chromophilic elements and presence of erythrocytes in narrow capillary sinuses (x 225).

Fig. 3. NORMAL pituitary tissue (x 225).

Schweizer *et al.*, 1940 (35), in a later publication containing the only available quantitative data on the subject, report a very marked adrenal atrophy after ocular pituitary grafting in the guinea pig. Unfortunately, in these studies, the morphological state of the adrenals was the only criterion of corticotrophic function. Our observation of adrenal atrophy concurrent with a stress-induced ascorbic-acid dis-

charge suggests that this is a somewhat inadequate index. The above two experiments show that such a discharge can still occur after complete severance of the pituitary from the diencephalic centers. This seems to exclude these centers as the necessary activators of the hypophyseal corticotrophic function and lends indirect support to the humoral theory. Yet it is possible that direct neural stimuli may also cause a corticotrophin discharge. There are no experimental data to support this view, although some clinical observations (36-39) seem to suggest it.

#### SUMMARY

In rats, severance of the hypothalamo-hypophyseal pathways was obtained in one experiment through partial hypophysectomy and in another, through pituitary homotransplantation into the anterior chamber of the eye. After these interventions, a significant discharge of adrenal ascorbic acid was still observed to result from surgical trauma and exposure to cold. It is concluded that the stimulation of corticotrophin release by cold or surgical trauma is not necessarily mediated through nervous pathways.<sup>2</sup>

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<sup>2</sup>Since this paper was submitted, a report has appeared (CHENG, C. P., G. SAYERS, L. S. GOODMAN, AND C. A. SWINYARD. *Am. J. Physiol.* 158: 45, 1949) which corroborates our results regarding the corticotrophic effect of stress after pituitary-stalk section. The possible regeneration of the hypothalamo-hypophyseal portal system, however, was not excluded through pituitary transplantation, but we understand, from personal communication with the authors, that such work is under way. [See preceding article—Editor.]

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# NORADRENALIN-LIKE SUBSTANCE IN BLOOD DEMONSTRATION BY CROSS-CIRCULATION

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THERE is evidence that vasoconstrictor substances appear in the blood of shocked animals (1) but it has not been determined whether or not these have vasopressor as well as constrictor effects. In normal animals and man, pressor substances in blood are occasionally encountered (2) but their function and the circumstances of their appearance are unknown. Clearly this inadequately studied field requires further investigation. It is the purpose of this work to ascertain by means of cross-circulation studies in dogs the occurrence of pressor substances after a variety of experimental procedures.

Since cross-circulation and perfusion experiments have been widely used to solve important problems, especially those in hemodynamics, a study of changes in the pressor properties of the cross-circulating blood has seemed overdue. Our results amply demonstrate that changes of great intensity may occur which would alter the results of supplemental experimentation. We have been unable to find reports of cross-circulation experiments in the literature in which this has been given adequate consideration.

The appearance of serotonin, the crystalline vasoconstrictor and vasopressor substance appearing in blood when it clots (3), could not be entirely avoided in all experiments, especially those in which a variety of operations had been performed. There is no doubt that small or even large clots could occur undetected in either of the animals in the cross-circulation circuit. To minimize them, large doses of heparin were repeatedly given during the course of most of the experiments. Despite this, serotonin might have contributed something to the vasoconstrictor and vasopressor action of some of the blood.

As our experiments show, another pressor substance appeared not only in shocked animals but in those subjected to a variety of operative traumata without overt shock. However, it was not limited to these, occurring also sporadically for short times in normal anesthetized (pentobarbital) or unanesthetized dogs. The results further show that the substance is neither renin nor angiotonin from the kidneys, adrenalin from the adrenal glands or serotonin associated with coagulation of blood. The evidence suggests it to be a noradrenalin-like substance, if not noradrenalin itself. This question cannot be finally settled at present, despite the strongly suggestive pharmacological evidence.

## METHODS

Intravenous pentobarbital anesthesia was employed in most experiments. In some, anesthesia

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was not necessary, especially those in which hepatectomy or adrenalectomy had been performed and those in which the cord had been destroyed.

Blood pressure was recorded on kymographs from mercury manometers connected to the femoral arteries. The dogs were heparinized to prevent blood coagulation and heparin solution was used in the plastic tubing which connected the venous circulation of the two dogs. Cross-circulation was performed by 50 cc. syringes inserted into a circuit connecting the jugular with the femoral veins. The withdrawal and reinfusion of blood were kept equal and consisted of the crossing of from 600 to 1000 cc.

We have tried pumps of the rotary squeegee type but found much hemolysis due to rupture of the cells. Further, we avoided arterial circuits because of their disturbing influence on blood pressure. The hand method proved satisfactory for our purposes, though it required two people to conduct it.

The operative procedures were all performed under sterile conditions. Bilateral nephrectomy was done a day or more before the experiment, and cord destruction from  $C_6$  caudad also a day or two before. On the day of the experiment, the carotid sinus was inactivated and the vagus nerves cut. Hepatectomy was performed by the method of Firor and Stinson (4) usually two or three hours before the experiment. Treatment of these animals followed the usual procedure as developed in the classic work of Frank Mann. Hypertension was produced by the silk perinephritis method of Page (5).

It should be emphasized that except when the persistence in the circulation of a substance was under study, nothing was given which might interfere with the effects of the cross-circulation itself.

Dibenamine<sup>1</sup> was given in doses from 10 to 20 mg/kg. body weight and priscol 1 to 5 mg/kg. Adrenalin as a test substance was given into the femoral vein in a total dose of 0.001 mg. in 0.2 cc. saline, and D-L-noradrenalin 0.04 mg. Tetraethylammonium chloride (TEA) was given in doses of 10 mg/kg. body weight. Scalding was done under deep pentobarbital anesthesia as we have described before (6). Tourniquets were placed on both hind legs and kept on for 2 to 4 hours. These were pulled as tightly as possible by hand, but were not completely occluding since great swelling of the limb occurred below the tourniquets. Renin was prepared by the method of Helmer and Page (7) and diluted to a point at which 0.1 cc. gave a rise of 40 mm. Hg in a normal dog under pentobarbital anesthesia.

In the tables, the data left of the vertical dividing line are those derived from dogs subjected to a variety of experimental surgical procedures, while those on the right are from the 'indicator' or 'test' dog. We have tended to call the first the donor dog and the opposite partner the 'indicator' or 'test' animal, although cross-transfusion was equal in both directions. For convenience, we shall, for example, use the abbreviated but grammatically peculiar expression, 'cord destruction-carotid sinus inactivation-nephrectomy to nephrectomy', meaning that the spinal cord was destroyed in one dog from  $C_6$  caudad, the carotid sinus tied off, the vagus nerves cut and bilateral nephrectomy performed one or two days before, and this dog was cross-transfused with another subjected to nephrectomy.

### *Results of Cross-Circulation*

*Normal to normal.* Seven experiments were made in which up to 5 liters of blood were crossed (table 1, *exper. 432*). No effect was usually observed on either dog's blood pressure, though occasionally a moderate rise or fall occurred, especially if the arterial pressure of one of the dogs had fallen. TEA injection may aid in enhancing what might have been small responses.

*Normal to nephrectomy.* Since dogs nephrectomized a day or two before the experiment usually exhibited heightened sensitivity to chemical stimuli, 9 experiments were done to determine whether or not nephrectomy augmented the action of any vasoactive substances which might be transferred by cross-transfusion (table 1, *exper. 544*).

<sup>1</sup>We are indebted to Dr. William M. Swain (Smith, Kline & French Laboratories) for the dibenamine, to Dr. Frederick Yonkman (Ciba Pharmaceutical Products) for the Priscol, and to Dr. R. C. Pogge and Mr. Z. M. Gibson (Merck & Co.) for the benzodioxane.

Except in occasional cases, the normal animals showed no rise in blood pressure while the nephrectomized ones usually did, especially after the first cross transfusion, even though the blood pressure of the normal donor was not reduced. As will be shown later, pressor substance tends to appear in the blood if hypotension or shock occurs.

*Splenectomy-resection and anastomosis of jejunum; temporary clamping vena cava and portal vein to nephrectomy.* It seemed desirable early in the investigation to ascertain whether extensive surgical operations as such would influence the results. Fourteen experiments were done in which the spleen was removed, about 2 feet of jejunum removed and an end to end anastomosis performed, and finally the vena cava and portal veins clamped for 3 minutes. The assumption is made that these procedures were traumatizing but not highly specific, which may not be true. When splenectomy was omitted, the results were not different. These consisted of a quite regular pressor response of the indicator nephrectomized dog with little in the traumatized donor dog (table 1, *exper. 501*). Large doses of TEA often seemed to augment the response.

*Nephrectomy-adrenalectomy to nephrectomy.* Pressor responses occurred fairly regularly in the nephrectomized indicator dog in 26 of the 27 experiments, demonstrating that the adrenal glands are not the sole, if indeed, an important source of the pressor agent. It was repeatedly noted that, contrary to experiments in which the conditions were less drastic, blood from the nephrectomized animal usually gave a depressor response in the nephrectomized-adrenalectomized dogs.

In the experiment used to illustrate this group (table 1, *exper. 633*), after three cross-transfusions, two of which gave rises in arterial pressure, another nephrectomized dog was substituted for the original indicator animal. Possibly this may have aided in keeping up the good responses. But it does not follow that the fresh indicator animal was necessary for the maintenance of response, as other experiments show that gradual loss of responsiveness may more probably be due to exhaustion of pressor substance in the donor rather than lack of responsiveness of the recipient. Administration of TEA after the response to the cross-circulation was lost, augmented twofold, at least, the response to injected adrenalin; but cross-circulation still gave little or no response.

Crossing of 600 cc. of blood from a nephrectomized-adrenalectomized dog into a nephrectomized animal resulted in a rise, for example, in one case, of 66 mm. Hg in the recipient and no change or a fall in the donor, when the arterial pressure in the donor was of the order of 50 to 80 mm. Hg. This is not unusual in such severely traumatized animals. Repeating the cross-circulation three or four times resulted in progressive loss of recipient response until none occurred. Injection of renin at this point showed the animal to be responsive to it. Tachyphylaxis to renin had thus not occurred, which is what might be expected from blood of a nephrectomized donor unless renin from some other source than the kidneys was present.

Such experiments as these suggest that the main source of the pressor substance is neither the kidneys nor the adrenal glands, and that repeated cross-circulation can either exhaust or dilute the pressor substance sufficiently so that it will fail to elicit a response in the recipient.

TABLE I. EXAMPLES OF CROSS-CIRCULATION EXPERIMENTS. RESPONSE OF ARTERIAL PRESSURE IN MM. HG TO ADRENALIN, TEA AND CROSS-CIRCULATION

EXPERIMENTAL DOG					INDICATOR DOG			
Adrenalin	TEA	B.P.	Amt. of blood crossed <sup>1</sup>	Response, mm. Hg	Adrenalin	TEA	B.P.	Response, mm. Hg
<i>Exper. 432</i>					<i>Normal</i>			
		148	540	0			106	0
		120	1540	0			110	0
	-70	114						
	-8	58	2080	0			104	0
		80	2620	0		-28+48	102	
		88	3160	0		+98	62	34
		104	3700	28			60	0
		128	4240	18			88	0
		144	4780	0			96	0
							94	0
<i>Exper. 544</i>					<i>Nephrectomy</i>			
10		138	600	0	42		108	54
14		130	1200	0	26		86	28
18		114	1800	0	10		146	14
14		112	3300	8	42			32
26		126	3900	0	64		90	0
<i>Exper. 501</i>					<i>Nephrectomy</i>			
6		102	600	0	60		154	40
		56	1200	0			142	62
10		60	1800	0			160	40
		78	2400	0			180	50
		82	3000	0			208	42
		86	3600	0			156	24
		98	4200	8			160	8
						168	172	—
		100	4800	0	194		136	44
<i>Exper. 633</i>					<i>Nephrectomy</i>			
	<i>Nephrectomy-Adrenalectomy-Burn</i>							
8		154	600	-24	20		64	-8
12		150	1200	10	14		66	18
		152	1800	-34	24		94	34
	<i>Changed to Another Nephrectomized Dog</i>							
		84	600	0	42		166	38
		98	1200	0			188	8
		80	1800	0	48		170	24
		50	2400	0			176	26
		20	3000	0			194	36
<i>Exper. 480</i>					<i>Nephrectomy</i>			
		118	600	14			124	30
		122	1200	0			146	0
		122	1800	0			120	34
		110	2400	0			140	10
						+8-44		
		98	3000	-50		+6-12	114	
		134	4200	0		-8	106	32
							144	38

<sup>1</sup> This represents total blood cross-circulated rather than each individual cross-circulation.

*Hepatectomy to normal.* The only thing noteworthy about these five experiments was that in one half the cross-circulations, blood from the normal dogs caused a sharp fall in blood pressure of the hepatectomized dogs. In only one normal dog, after large doses of TEA was a questionable rise in arterial pressure observed after cross-circulation.

*Nephrectomy-hepatectomy to normal.* Except toward the end of the experiments, little or no pressor responses were observed. As the experiment progressed, either dog might exhibit elevation of pressure on cross-circulation, but such changes were not large and were irregular.

*Nephrectomy-hepatectomy to nephrectomy.* In 15 experiments, cross-circulation from nephrectomized-hepatectomized dogs into animals nephrectomized one or two days before caused rises in blood pressure chiefly in the nephrectomized indicator dog. Often the nephrectomized-hepatectomized dog also exhibited pressor responses, especially toward the end of the experiment (for example table 2, *exper. 420*). TEA administration did not cause the appearance of pressor responses when they had not been there previously. The results show clearly that neither the liver nor kidneys are necessary for the pressor responses to appear on cross-circulation with a nephrectomized dog. Indeed, in these experiments, the responses in general appear to be somewhat greater than when the liver is intact, though this cannot be considered proved.

*Nephrectomy-hepatectomy to hepatectomy.* No striking responses were seen in either dog on cross-circulation in the one experiment which was satisfactory.

*Hepatectomy to nephrectomy.* Large pressor responses were observed in 3 experiments in the nephrectomized dogs and little or none in the hepatectomized animals.

*Cord destruction-carotid sinus inactivation-nephrectomy-dibenamine to nephrectomy.* Enough dibenamine was given to 15 animals highly sensitized by cord destruction and carotid sinus inactivation, to reverse the adrenalin response but not the response to noradrenalin. Cross-circulation from the nephrectomized dogs still produced a rise in pressure in the sensitized dogs and often a fall in the nephrectomized animals (table 3, *exper. 790*; also fig. 2). If enough prisol was also given to reduce or abolish the response to noradrenalin, the pressor response from cross-circulation appeared to be reduced or abolished in parallel fashion (table 3, *exper. 801*). Normal dogs used as donors also caused marked pressor responses (table 3, *exper. 758*) even when the adrenalin pressor response was abolished.

*Cord destruction-carotid sinus inactivation-nephrectomy to nephrectomy-hepatectomy.* In none of the 4 satisfactory experiments did cross-circulation cause a rise of pressure in the nephrectomized-hepatectomized donor, but all of the sensitized recipients exhibited significant elevation of pressure. The rise was not abolished when the pressor response of adrenalin was reversed by prisol, but was reduced or abolished when the noradrenalin response was sharply curtailed by injection of both prisol and dibenamine (table 3, *exper. 827*).

*Cord destruction-carotid sinus inactivation-nephrectomy to hepatectomy-nephrectomy-adrenalectomy-splenectomy.* No pressor action was observed during cross-circulation in the dogs with liver, adrenal glands, kidneys and spleen removed. But in the 4 sensitized animals, the responses were clearly pressor (table 3, *exper. 839*).

*Scald-nephrectomy to nephrectomy.* Cross-circulation of nephrectomized-scalded

dogs with nephrectomized animals did not produce a rise in arterial pressure in any of the 9 experiments in the donor, while the nephrectomized indicator dog usually exhibited significant and repeated rises. About a quarter of the cross-circulations indeed produced a fall in arterial pressure in the scalded animals. Even when a normal dog was substituted for the nephrectomized one as indicator, pressor responses were still obtained and showed no signs of developing tachyphylaxis (table 3, *exper.* 662).

TABLE 2. CROSS-CIRCULATION OF NEPHRECTOMIZED-HEPATECTOMIZED DOG WITH A NEPHRECTOMIZED DOG (*Experiment 420*)

NEPHRECTOMY-HEPATECTOMY						NEPHRECTOMY			
Adrenalin	Renin	TEA	B.P.	Amt. Crossed	Response	Renin	TEA	B.P.	Response
					mm. Hg				
20	52		152						
	0		180	900	0			140	64
22		-72	162						
		-6	114						
	20	0	116						
62	0		124	1440	0			170	20
	0	0	120	1980	0			180	12
	0	0	134	2880	0			186	14
			136	3420	0	14		180	0
							+6-10	186	
							+30	176	
			152	3960	0		+10	162	52
	8		160	4500	0		0	156	44
			176	5040	0	0	0	160	20
			176	5580	0			146	16
			174	6120	0		0	144	0
			172	6660	0	0	0	130	16
	20		170	7200	0		0	132	28
	18		176	7740	0			132	38
	10		190	8280	74	0		130	32
			180	8820	-16		0	140	16
	12		186						
26	0		164	9600	40				

*Scald-nephrectomy-adrenalectomy to nephrectomy.* As in the experiments just described, no pressor response to cross-circulation was noted in the 9 scalded dogs in which the kidneys and adrenal glands had both been removed. But the nephrectomized animals exhibited well sustained rises during the cross-circulation. Since the results of these experiments are so similar to those in which the adrenal glands were not removed, examples will not be given in the tables.

*Scald-nephrectomy-adrenalectomy to cord destruction-sinus inactivation-nephrectomy-priscol.* In these experiments, the response of the highly sensitized (cord destroyed) dogs were sharply pressor to cross-circulation with the scalded nephrectomized-adrenalectomized dogs. When the sensitized dogs were given enough priscol

to reduce significantly the pressor response to 1-noradrenalin, then cross-circulation gave little or no response (table 3, *exper. 851*). If a fresh cord-destroyed dog was substituted for the prisol-treated one, a pressor response was again obtained.

*Tourniquet-nephrectomy to nephrectomy.* This series of 11 experiments was similar in conduct to those using scalding, hence will not be presented in detail. The data show that pressor responses are usually observed on cross-circulation and only occasionally are seen in the nephrectomized dogs with tourniquets. It seems to make little difference whether the tourniquets have been loosened or not. The blood from the nephrectomized dog is often depressor when cross-circulated into the tourniquet-nephrectomized animal.

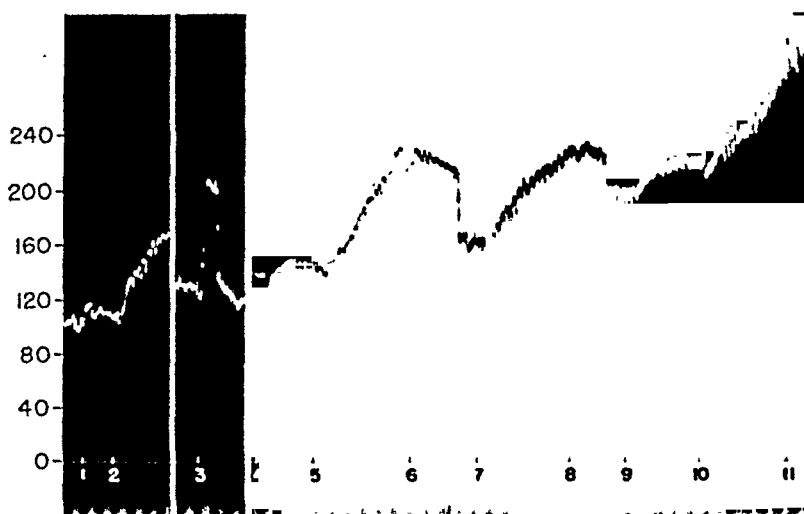


Fig. 1. EXAMPLE OF PRESSOR EFFECT of cross-circulated blood from nephrectomized dog into nephrectomized-hepatectomized dog (tracing shown). The experiment is unusual in that augmentation of the adrenalin response by TEA was much greater than usual. 1, Usual weak response of hepatectomized dogs to adrenalin; 2, usual good response to renin when the kidneys are removed before hepatectomy is performed; 3, later response to adrenalin after large doses of TEA were given; 4, poor response to renin despite TEA.; 5-6, cross-circulation of 540 cc. blood; 7-8, second cross circulation; 9, renin; 10-11, third cross circulation. The tendency for the average blood pressure to rise after repeated cross circulation is well illustrated.

Occasionally, when the pressor material seemed exhausted, the abdominal cavity was opened and the intestines stripped, or the animal subjected to repeated hemorrhage. Often, but not always, this led to reappearance of pressor substance in the blood. Much the same results were observed when blood was cross-circulated from a tourniquet-adrenalectomy-nephrectomized dog to one with kidneys removed and the spinal cord destroyed. Prisol in doses large enough to reduce the noradrenalin response also reduced the pressor response to cross-circulation (table 3, *exper. 863*).

*Renal hypertensive to cord destruction-sinus inactivation-nephrectomy.* Renal hypertension was produced a month or more before these 15 experiments by wrapping both kidneys in silk (5). Dogs sensitized by nephrectomy carotid sinus inactivation and cord destruction were used to indicate pressor effects of the cross-circulated blood.

Pressor responses were observed after all but one cross-circulation in the indicator dogs, but none occurred in the hypertensive dogs (table 3, *exper. 722*). Rather, a

moderate fall in pressure often was observed. While the pressor responses as the result of cross-circulation of hypertensive dogs seem to be more regular than in most of our other experiments, the differences were not great enough to indicate a fundamentally different or omnipresent pressor system in renal hypertensive animals.

Dibenamine and priscol reduced or abolished the response in doses which did not block renin pressor action.

### *Transfer of Vasopressor Substances*

Many experiments show that, except under unusual circumstances, injected adrenalin in single doses does not persist in the circulation long enough to be transferred to another animal. We have only been able to demonstrate it by injecting large amounts and immediately cross-circulating. For example, 0.3 cc. of 1:1000 adrenalin was injected into the femoral vein of a nephrectomized dog and cross-circulation with another nephrectomized dog immediately started. The blood pressure rose 110 mm. Hg in the donor but after the cross-circulation of 330 cc. of blood within 3 minutes, the pressure of the nephrectomized recipient rose 42 mm. Hg. Noradrenalin in single doses, on the other hand, at times may be transferred often for several minutes after its injection, especially into nephrectomized animals. More usually, it has disappeared from the circulation when the blood pressure effect is over. On several occasions, when the dog was *in extremis*, the effect persisted in the plasma for periods up to 10 minutes. Blood in 25 to 50 cc. amounts removed during that period, when injected into a fresh test dog, exhibited sharp rises in arterial pressure, which could be abolished by doses of priscol or benzodioxane sufficient to abolish the response to noradrenalin. If instead of single doses, noradrenalin is infused at rates sufficiently great to produce a sustained rise of 100 mm. Hg or more, the transfer is more readily accomplished. The response curve in the indicator animal is indistinguishable from that resulting from cross-circulation and is obliterated by large doses of priscol-benzodioxane.

Renin transfer is more irregular and difficult to predict. If a normal dog is made tachyphylactic to renin by its repeated injection, and cross-circulation with a nephrectomized dog immediately started, a rise of 60 or more mm. Hg may be observed in the recipient animal. A second cross-circulation 20 minutes later, while the donor is still tachyphylactic, produces no further rise in the indicator dog's pressure, despite the continued responsiveness of this animal to directly injected renin.

Even in some hepatectomized-nephrectomized animals, renin does not persist in such form as to be active when transferred to a nephrectomized recipient for more than a few minutes. For example, in one experiment, two doses of renin made the hepatectomized-nephrectomized dog tachyphylactic. Cross-circulation of the 540 cc. of blood with a one-day nephrectomized dog gave a rise of 48 mm. Hg in the latter and no change in the donor. Five times as much renin was then given as initially injected, with only a 20 mm. rise and 7 minutes after its injection, cross-circulation with the nephrectomized indicator dog was instituted. No change in the pressure occurred in the latter.

This result, however, cannot always be counted upon. The following experiment illustrates what appears to be the occasional persistence of renin, ready to react

TABLE 3. RESPONSE OF ARTERIAL PRESSURE TO ADRENALIN, NORADRENALIN AND CROSS-CIRCULATION

EXPERIMENTAL DOG					INDICATOR DOG				
Adrenalin	Nor-adrenalin	Blood Pressure	Cross-Circulated Blood Amount in cc. <sup>1</sup>	Response in mm. Hg	Adrenalin	Nor-adrenalin	Blood Pressure	Response in mm. Hg	Exper. No.
<i>Cord Destruction-Carotid Sinus Inactivation-Nephrectomy to Nephrectomy</i>									
106	64			Dibenameine given	24		136		790
-18		100	560	20	24		120	-28	
		110	1760	56	28		104	6	
		150	2560	20	20		126	0	
	76	174	3360	0			138	0	
		176	6080	56			140	0	
	74	108	8160	62	10		50		
-84	18	106	10,600	36	12		60	-40	
<i>Dibenameine Given Before Experiment</i>									
8	22	56	920	0	32	40	140	4	801
	28	70	2920	30	6		160	-34	
+40-10	64	98		Priscol given	20		120		
-16	0	44	3160	0			130	0	
	30	50	5000	24			150	-84	
-30	4	44	7000	-2			62	-40	
<i>Normal to Cord Destruction-Carotid Sinus Inactivation-Nephrectomy</i>									
34		140	2000	-20	+18-16		154	80	758
		108	3000	-12			98	46	
		94	6000	-8			96	146	
<i>Nephrectomy-Hepatectomy to Cord Destruction-Carotid Sinus Inactivation-Nephrectomy</i>									
10	14	80	0	126	132	66	480	38	827 After control responses priscol then given dibenameine
		62	0	10	54	92	1280	1	
		58	0	12	48	66	2080	6	
<i>Nephrectomy-Hepatectomy-Adrenalectomy-Splenectomy to Cord Destruction-Nephrectomy</i>									
-22	28	72	0	100	132	106	900	16	839
-16	22	70	0	90	114	108	1280	46	
		64	0			118	1680	38	
		62	0			126	2080	18	
<i>Scald-Nephrectomy to Nephrectomy</i>									
22		144	600	-12	32		120	-54	662
22		132	960	0	26		124	16	
		142	1320	0			140	0	
		144	1980	0	72		138	22	
		144	2340	-6			152	12	
		142	2700	0	64		154	26	
		144	3060	0			158	22	
		144	3660	0			158	18	
		140	4020	0			160	12	
		94	4380	-22			154	70	
		76	4700	-8			156	46	

TABLE 3.—Continued

EXPERIMENTAL DOG					INDICATOR DOG				
Adrenalin	Nor-adrenalin	Blood Pressure	Cross-Circulated Blood Amount in cc. <sup>1</sup>	Response in mm. Hg	Adrenalin	Nor-adrenalin	Blood Pressure	Response in mm. Hg	Exper. No.
<i>Scald-Nephrectomy-Adrenalectomy to Cord Destruction-Nephrectomy</i>									
26		114	480	0	64	110	84	74	851
36		80	1280	0		126	116	30	
12			1800	0		134	118	34	
26		70	2280	0		110	116	12	
20			3360	0	16	24	100	0	
6		48	1200	0	40		78	18	Fresh cord dog substituted
<i>Tourniquet-Adrenalectomy-Nephrectomy to Cord Destruction-Nephrectomy</i>									
30	48	92	480	0	106	132	68	48	863
26	46	74	800	0	108	130	120		
20		64	1200	0			98	38	
18		50	1600	0		168	114	38	Prisol 7.5 mg/kg.
14	22	62	2600	0	+16-26	32	102	14	
8		62	3400	0		12	84	16	
<i>Hypertensive to Cord Destruction-Nephrectomy</i>									
22		192	1000	10	100		160	16	722
		210	2000	0	100		148	26	
20		204	2450	-10	90		154	20	
		194	3200	0	84		166	10	
		180	4200	-16	108		138	22	

<sup>1</sup> This represents the total amount of blood cross-circulated rather than individual cross-circulations.

whenever the chemical environment is favorable. It will be recognized that the substance we transfer need not necessarily be renin, even though large amounts had been injected into the donor. A nephrectomized, TEA-treated dog was made tachyphylactic to renin and 10 minutes later 600 cc. of blood was crossed with a nephrectomized dog. A rise of 92 mm. Hg occurred in the latter and 28 mm. Hg in the renin-treated donor. Five minutes later a similar cross resulted in a rise of 46 mm. Hg in the recipient and no change in the donor. Renin was then repeatedly injected into the recipient until the responses were sharply reduced and the lack of response of the donor assured by injecting more renin. Crossing then gave no response in either animal. We have repeatedly observed that when both animals are made tachyphylactic to renin, the chances of observing a pressor response in the animals from whatever the substance in the cross-circulating blood are reduced.

The reason we think it doubtful that all of the active pressor material carried over is renin can be illustrated by the following experiments, 14 of which were done. Four doses of renin were given a hepatectomized-nephrectomized dog and 3 doses of TEA (2.5 mg/kg.). The last dose of renin gave no pressor response. Three minutes

later, 540 cc. of blood was cross circulated within 4 minutes with a nephrectomized dog. No pressor effect was observed in the donor (hepatectomized-nephrectomized) and only 34 mm. Hg in the untreated indicator animal. Without the injection of renin into the donor, such a rise from cross-circulation, especially the first one, was to be expected. In another experiment, large doses of renin and TEA were given to a nephrectomized-hepatectomized donor, without eliciting a significant response. Four minutes after the first cross-circulation, the last injection of renin gave a response in the indicator nephrectomized dog of 74 mm. Hg. Still more renin was given the donor and 6 minutes later another cross-circulation was performed. Now only a 18 mm. Hg rise was noted. Even when no response occurred from cross-circulation, injection of renin into the nephrectomized recipient still gave a pressor response.

In normal dogs, 9 to 19 injections of renin given in the course of 3 hours produced complete tachyphylaxis. Cross-circulation with a normal dog was without effect on the blood pressure of either animal. Since this result is regularly obtained, it is unlikely that significant amounts of injected renin, in active form, circulate in normal animals for at most more than a few minutes.

Occasionally, TEA seemed to be carried over during cross-circulation when started not more than a minute or two after injection of the drug. Like adrenalin, its occurrence in the blood in significant quantity appeared to be short-lived.

#### *Augmentation by TEA*

We demonstrated several years ago that TEA in doses of 10 to 20 mg/kg. body weight augmented the vascular responsiveness to a variety of chemical substances (8, 9). It was especially active in the case of adrenalin and noradrenalin.

Augmentation of the pressor response to cross-circulation has not been proved beyond doubt because of the inability to know whether the same amount of pressor agent is delivered with each cross-circulation. Indeed, most of our evidence strongly suggests progressive reduction in the amount delivered, because the substitution of a fresh, nephrectomized dog as an indicator animal seldom produces any greater response than the indicator animal initially used. If the test dog had become refractory or tachyphylactic, this would have been easily demonstrated.

Augmentation of the pressor responses after TEA was often observed, especially when only small cross-circulations had initially been made. The augmentation is irregular and unpredictable. The evidence cannot, therefore, be used for or against similarity with noradrenalin. It merely suggests that the supply of pressor agent available at each cross-circulation is limited.

#### DISCUSSION

These experiments show clearly that pressor substance may appear in the heparinized blood of dogs, demonstrable by cross-circulation into another dog. Neither its appearance nor detection is dependent on the pentobarbital anesthesia used in part of the experiments, because in unanesthetized animals with spinal cords destroyed, the pressor substance was also easily shown.

The highly sensitized dog, i.e. with spinal cord destroyed and carotid sinus mechanism inactivated, was the one most likely to indicate pressor substance and,

therefore, it was to be expected that such animals would show the highest incidence of pressor reaction on cross-circulation. Two-day nephrectomized dogs, especially if given 200 cc. saline subcutaneously daily, were also highly sensitive. As we have shown (9) sensitivity is even further increased by cord destruction and carotid sinus inactivation.

Using these highly sensitive animals as indicators of pressor activity, cross-circulation of from 500 to 1000 cc. of blood over a period of from 5 to 7 minutes often resulted in a rise of 50 to 75 mm. Hg arterial pressure in the sensitized dog. The rise persists during the period of cross-circulation, often with little or none in the opposite



Fig. 2. EXPERIMENT DEMONSTRATING that the pressor agent is still active after blockade of adrenalin with priscol. Doses of priscol sufficiently large to block noradrenalin also block the rise in arterial pressure from cross transfusion. The graph illustrated is from the nephrectomized indicator animal cross circulated with a nephrectomized-adrenalectomized dog. 1, adrenalin; 2-3, cross circulation 540 cc. blood; 4, TEA 5 mg/kg.; 5, adrenalin; 6-7, cross circulation; 8, priscol, 5 mg./kg.; 9, adrenalin; 10-11, cross circulation; 12, adrenalin (*exper. 577*).

partner in the circuit. The results of our cross-circulation experiments may be summarized as follows:

#### *Summary of Pressor Responses to Cross Circulation*

1. Normal  $\pm$  to normal  $\pm$
2. Normal  $\pm$  to nephrectomy  $++$
3. Splenectomy-resection of jejunum  $+$  to nephrectomy  $+++$
4. Nephrectomy-adrenalectomy to nephrectomy  $++++$
5. Hepatectomy  $+$  to normal  $\circ$
6. Nephrectomy-hepatectomy  $\circ$  to normal  $\circ$
7. Nephrectomy-hepatectomy  $+$  to nephrectomy  $++$
8. Nephrectomy-hepatectomy  $\circ$  to hepatectomy  $\circ$
9. Hepatectomy  $\circ$  to nephrectomy  $+++$
10. Cord destruction-sinus inactivation-nephrectomy  $+++$  to nephrectomy  $++$
11. Cord destruction-sinus inactivation-nephrectomy  $++$  to nephrectomy-hepatectomy  $\circ$
12. Cord destruction-sinus inactivation-nephrectomy  $+++$  to nephrectomy-hepatectomy-adrenalectomy-splenectomy  $\circ$
13. Scald-nephrectomy  $\circ$  to nephrectomy  $++++$
14. Scald-nephrectomy-adrenalectomy  $\circ$  to nephrectomy  $+++$

15. Scald-nephrectomy-adrenalectomy o to cord destruction-sinus inactivation, nephrectomy + + + +
16. Tourniquet-nephrectomy + to nephrectomy + +
17. Renal hypertension o to cord destruction-sinus inactivation-nephrectomy + + + +

When normal animals are cross-circulated, a rise occurs only occasionally, but if one nephrectomized animal is substituted, the rise becomes greater and possibly minor ones are augmented. Greater rises were also obtained after cord destruction and carotid sinus inactivation; hence dogs prepared in this way were usually employed to indicate the presence of vasoactive substances. If the adrenal glands, as well as the kidneys, are removed in a dog, pressor responses of significance are still obtained, demonstrating that neither the kidneys nor the adrenal glands are the sole source of the pressor material. The spleen seems to play a negligible part in the mechanism. Removal of the liver, as well as the kidneys, does not abolish the pressor response, though the results suggest a reduction of its frequency and intensity. If in addition, the adrenal glands and spleen are removed, the blood from such a preparation still gives pressor responses. These varied combinations of organ removal have demonstrated to us that certain organs not essential for the response, and further that sensitized preparations, such as the 'nephrectomized-cord' dog, are more likely to detect changes in the pressor qualities of cross-circulated blood than are the more nearly normal ones. Although the liver may supply some of the pressor material, it is not the only source and probably not the major one. Clearly the adrenal glands, kidneys and spleen are not essential for pressor responses.

Pressor responses still occur to cross-circulation when those to injected adrenalin have been reversed by prisclo and dibenamine; however, when enough of these agents are given to block L-noradrenalin, they are abolished. This does not prove that the pressor substance is noradrenalin, since other pressor agents are blocked, or the intensity of their action reduced, by such large doses of the blocking agents. But the fact that there is this parallelism of action, as well as the fact of its diffuse origin in the body, suggests that it is indeed noradrenalin.

Several years ago, we demonstrated in the plasma and plasma ultra-filtrate of scalded dogs the appearance of a vasoconstrictor substance (1). Later we suggested, chiefly by exclusion, the possibility that this substance might be noradrenalin (10). To investigate the matter further, dogs were subjected to scalding or tourniquet shock after nephrectomy, nephrectomy-adrenalectomy, etc., and cross-circulated with sensitized indicator animals. The blood from these animals was usually strongly pressor, and was comparable to that from some of the animals having had extensive surgical procedures.

Thus a direct proof is given that blood from shocked animals may be not only vasoconstrictor but vasopressor as well.

It is of interest that pressor responses failed to be observed in only one out of 13 dogs with severe renal hypertension (persisting longer than 6 weeks), when sensitized test dogs were used in the circuit. The significance of this observation cannot at present be adequately evaluated, although the observation is a striking one.

Repeated cross-circulation appears to exhaust the supply of pressor material temporarily. The reasons for this belief are the progressive elimination of pressor

response with repeated cross-circulation, the fact that the indicator animal in most cases did not show sufficiently reduced responses to injected adrenalin, noradrenalin or angiotonin to suggest that tachyphylaxis or refractoriness had developed, and finally, the fact that substitution of fresh indicator animals in the circuit did not restore pressor responses which were previously lacking.

TABLE 4. RESPONSE OF NEPHRECTOMIZED DOGS WITH CORD DESTROYED (C<sub>6</sub> ↓) TO CROSS CIRCULATION WITH RENAL HYPERTENSIVE DOGS

HYPERTENSIVE DOG B. P. 210 MM. HG (EXPER. 722)					CORD-NEPHRECTOMIZED DOG (NO ANESTHESIA)			
Drug	B. P. mm. Hg	Re- sponse	Amt. Cross- Circulated	Re- sponse	Drug	B. P.	Re- sponse, mm. Hg	Cross Circula- tion Re- sponse
Adrenalin	204	20			Adrenalin	140	90	
Adrenalin	188	22			Adrenalin	144	100	
Adrenalin	188	22			Adrenalin	148	98	
	182		1000 CC.	10		160		16
					Adrenalin	146	100	
	210		1000 CC.	0		148		26
	204		450 CC.	-10		154		20
	194		750 CC.	0		166		10
					Adrenalin	164	84	
					TEA 5 mg/kg.	158	32	
					TEA 5 mg/kg.	164	24	
					Adrenalin	168	108	
	180		1000 CC.	-16		138		22
Hypertensive Dog B.P. 220 mm Hg (Exper. 735)					Cord Destroyed Dog C <sub>6</sub> ↓			
Adrenalin	206	28			Adrenalin	140	68	
Adrenalin	198	26			Adrenalin	142	78	
	204		1000 CC.	0		128		64
					Adrenalin	162	58	
					TEA, 10 mg/kg.	154	32	
					Adrenalin	150	104	
	210		1000 CC.	10		164		20
					TEA, 5 mg/kg.	200	36	
					TEA, 5 mg/kg	210	20	
					TEA, 5 mg/kg	132	14	
					Adrenalin	140	142	
	220		1290 CC.	-10		120		38
	208		2430 CC.	-38		150		42

The problem of the rapidity with which injected vasoactive substances become inactivated and therefore not transferable by cross-circulation was an interesting one. Adrenalin in single doses was most quickly inactivated and, except when large doses were given, could not be demonstrated by cross-circulation after the rise in arterial pressure had subsided. Although L-noradrenalin was inactivated somewhat more slowly, it could not be detected after its pressor response, except in a few ex-

periments toward their end. In such cases, the kidneys and often the liver had been removed. But infusion into donor animals permits its cross circulation and response in the indicator animal with easy controllability. Barium chloride in the doses used to produce a rise of 30 mm. Hg could not be transferred and the same was true of angiotonin. TEA in large doses occasionally could be transferred, especially from a nephrectomized animal a few minutes after administration of the drug.

Success in the transfer of renin by cross-circulation was always difficult to predict. In most cases, no rise in blood pressure occurred in the recipient about 5 minutes after renin injection into the donor. In others, especially those in which the liver and kidneys had been removed, renin persisted for 10 minutes or more—30 minutes in one case. From the small amounts of renin required to make a nephrectomized-hepatectomized donor tachyphylactic and yet yield blood, which when crossed, is strongly vasoconstrictor, it is suggested that the liver is concerned with regulation of the level of circulating renin.

We had hoped to be able to demonstrate the occurrence of pressor substances in the blood of dogs with renal hypertension, since the test animals were far more sensitive than those formerly used. It appeared from the work of Solandt, Nassim and Cowan (11) that mechanical cross-circulation demonstrated the regular occurrence of greater than normal amounts of vasoconstrictor substance in the renal hypertensive animal's blood. Our results show the more regular occurrence of the noradrenalin-like substance in hypertensive dogs as compared with normotensive ones but the amounts indicate no obvious relationship to the degree of hypertension. One would expect that a substance causing the hypertension would be present constantly and be related to the severity of the hypertension. This is not true in our experience.

These experiments do, we believe, demonstrate not only the possible occurrence of such active substances as noradrenalin under a variety of circumstances but emphasize the need for the greatest care in interpreting experiments on the vascular system in which blood has either been cross-circulated or circulated through pumps, tubing, gauges etc. Not only can substances like serotonin form, but the noradrenalin-like ones as well may be liberated. From the large literature on perfusion, particularly as related to hemodynamic studies, we have so far found no investigation which has treated the perfused blood as though it were capable of exhibiting highly variable vasoactive properties. These experiments seem to us to show clearly that the variations may be great and are usually unpredictable. Without recognition of this fact, deductions based on an assumption of constancy of the preparation may be grossly erroneous.

We should not fail to recognize that this work, started with an entirely different purpose in mind, provides striking indirect confirmation of the thesis originally proposed and supported by a variety of experimental evidence by Walter Cannon and his school and more recently by von Euler, Bacq and Rosenblueth.

#### SUMMARY AND CONCLUSIONS

Repeated cross-circulation of from 600 to 1000 cc. portions of heparinized blood was performed between dogs which had various organs removed in an attempt to

demonstrate the presence of pressor substances in the blood. The animals used to demonstrate the occurrence of pressor substance ('indicator dogs') were made highly sensitive by bilateral nephrectomy, spinal cord destruction and carotid sinus inactivation.

The removal of liver, kidneys, spleen and adrenal glands, followed by cross-circulation with indicator dogs, shows that none of these organs is exclusively responsible for the appearance of pressor substances in the blood. Periods of shock and low blood pressure tend to increase the occurrence of pressor substance in the blood. The blood of dogs with experimental renal hypertension seems more apt to contain pressor substance than normal. Its occurrence is not sufficiently regular to suggest the maintenance of the hypertension by this type of pressor agent. Blood may become pressor under a variety of circumstances and is not limited to shock or hypertension. Dibenamine and priscol, in doses large enough to block the action of D-L-noradrenalin or L-noradrenalin, also block the pressor action of cross-transfused blood. Tetraethylammonium chloride augments the response.

It was demonstrated that infused noradrenalin can be transferred by cross-circulation with ease and the arterial pressure curve in the indicator animal is similar to that resulting from the naturally occurring pressor substance in blood. Adrenalin is much less easy to transfer. Single doses of adrenalin and noradrenalin disappear quickly from the blood and unless the amount given is very large are not transferable. Renin transfer is irregular; in normal animals it does not circulate in active form for more than a few minutes; in hepatectomized-nephrectomized ones it may persist for some time, suggesting the participation of the liver in the control of the blood level of renin.

A pressor substance is found in the heparinized blood of dogs under a variety of circumstances which may best be demonstrated by cross-circulation with sensitized dogs. It is believed to be noradrenalin, or at least noradrenalin-like, because *a*) it occurs even after a variety of organs have been removed, *b*) methods which sensitize to L-noradrenalin also do so for the pressor substance, *c*) methods which block L-noradrenalin similarly block the pressor substance, *d*) tachyphylaxis does not appear to occur and *e*) infused noradrenalin can be cross-circulated just as the pressor substance.

Both shock and experimental renal hypertension increase the incidence of the appearance of demonstrable amounts of noradrenalin in the blood but neither is a necessary circumstance. A variety of less abnormal stimuli produce it but apparently with less regularity.

The appearance of sufficient quantities of noradrenalin in the blood stream to be demonstrable by a rise in pressure after cross-circulation into another animal is not a regular occurrence, rather it appears to be a response to unusual and trying circumstances, among which may be included shock and renal hypertension.

The need for control of the appearance of substances such as noradrenalin and serotonin in perfusion and cross-circulation experiments is pointed out.

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# DOUBLE ACTION OF ADRENALIN ON THE SMALL INTESTINE<sup>1</sup>

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**A**DRENALIN, in addition to inhibiting movements of the gut through its action on the sympathetic nerves, also stimulates the gut muscle under certain conditions. This was first observed by Johannes Müller (1) in 1837, and subsequently by others in the intact animal. In the isolated gut Sharpey-Schafer (2), Tashiro (3) and Hoskins (4) obtained stimulation from small doses of adrenalin, and Hoskins (4), Lucas and Bonnycastle (5) obtained stimulation after washing away a large dose. Stimulation by adrenalin has been reported after eserine (6) and after pilocarpine (7, 8). This paper presents work on the mechanism of this stimulating action.

## METHODS

Experiments were made mainly on rabbits. Animals were kept on full diet prior to death and were killed by a blow on the neck followed by bleeding. A segment of the small gut was carefully excised and kept in Tyrode's solution for subsequent use. A suitable piece from this segment about 2 to 3 cm. in length was chosen and suspended vertically in a simple plain muscle bath containing Tyrode's solution, volume 30 cc., at 38°C. Oxygen was run through a specially made conduit tube so that the bubbles came out too small to disturb the movement of the gut. Movements of the longitudinal and circular coats were recorded simultaneously on the same drum, the former by a frontal writing point and the latter (upper one) with a McDowall recorder (modified air displacement method, 9). The upward and downward movements represented contraction and relaxation, respectively. A suitable arrangement was made to wash out the bath from below with as little disturbance as possible. Drugs were added quickly in solution made up in distilled water to the bath away from the tissue so that it became mixed immediately with the bath solution by the oxygen bubbles. Drugs used were adrenalin, physostigmine salicylate, and atropine sulfate.

## RESULTS

A fresh piece of small gut in a bath of Tyrode's solution showed stimulation on the addition of adrenalin to a concentration of  $10^{-8}$  to  $10^{-11}$  (fig. 1). Atropine abolished it. The extent of this stimulation is so small that it is difficult to demonstrate, and hence likely to be confused with the undulatory movements normally exhibited

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by the small intestine. Moreover, the variable responses of the gut to atropine from inhibition to stimulation give rise to genuine difficulty in such experiments on the gut.

Stimulation of the gut took place when an inhibitory dose of adrenalin was washed away with Tyrode's solution (fig. 2). Due care was taken to prevent variation in temperature of the bath during the two washings and notice was taken of the change in the movement of the gut when the bath was emptied. Atropine abolished this stimulation. Stimulation of the gut was also obtained by an inhibitory dose of adrenalin when eserine preceded it. *In vitro*, the stimulation by eserine alone was a short-lasting one, if air was passed through the bath instead of oxygen. In the presence of oxygen it was a prolonged one and thus the stimulation by adrenalin was difficult to demarcate. Atropine abolished this stimulation.

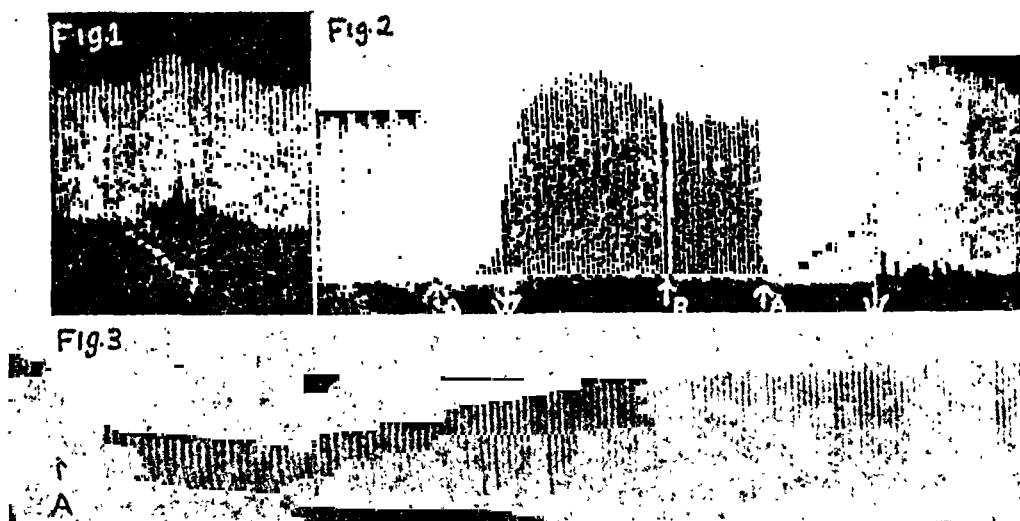


Fig. 1. RABBIT'S ILEUM. Stimulation by a small dose of adrenalin, 1 in  $10^{-8}$  to  $10^{-11}$   $\mu\text{g.}$ , at A. Bath volume 30 cc. of Tyrode's solution.

Fig. 2. RABBIT'S ILEUM. Upper tracing records volume; lower tracing records the longitudinal coat. A = adrenalin (10.0  $\mu\text{g.}$ ), which was washed away at W. R = rest. Adrenalin was allowed to act for different times.

Fig. 3. RABBIT'S GUT. Recovery and overaction after an inhibitory dose of adrenalin (10.0  $\mu\text{g.}$ ) in a Tyrode bath of 30 cc. at A.

Adrenalin stimulated the gut after a dose of acetylcholine. It was found to be essential to adjust the interval between the addition of the drugs. When the interval exceeded a particular limit the gut instead of showing stimulation showed enhanced inhibition.

The foregoing experiments were repeated in exactly similar ways on gut cooled in Tyrode's solution for more than 48 hours when no stimulation was found but only inhibition.

A sufficiently nicotinized piece of gut as judged by the absence of stimulation by a fresh addition of nicotine (100  $\mu\text{g.}$ ) yielded the same results as in the case of the cooled gut.

When a dose of adrenalin sufficiently large to produce inhibition was added and the gut allowed to remain in the bath in contact with adrenalin, the gut showed spontaneous recovery after some time and in some instances an overaction (fig. 3).

## DISCUSSION

From the experimental results it is clear that adrenalin has both a stimulating and an inhibitory effect on the gut depending upon the dosage (4). The inhibitory effect by a large dose predominates and masks the stimulating action of the small dose. In attempts to determine the cause of stimulation, previous investigators (4, 5) considered a number of possibilities, both chemical and physical. The stimulation after a wash was also explained on mechanical ground, and ultimately called a "paradoxical washout phenomenon." The finding herein reported is different.

It should be noted that previous workers obtained stimulation after washing away an inhibitory dose of adrenalin with Fleisch's, Dale's and Ringer's solutions, and in the present experiments with Tyrode's solution. Thus the stimulation cannot be attributed to the specific effect of a particular solution or its constituents. Consequently, this stimulation may be considered to be the same as by a small dose.

The salient feature is that the stimulation occurring under the various conditions detailed herein always took place in the fresh gut, i.e. when the gut had its tone and never in a gut cooled for more than 48 hours or after nicotization, both of which cause a fall in the tone. Krishnan (8) recorded stimulation of the gut by adrenalin after its tone was heightened by eserine, pilocarpine and acetylcholine. It may be recalled that both the processes of cooling (10, 11) and nicotization (12) are known to abolish nerve action. From this it appears that stimulation of the gut is related to the functionally active intrinsic nerve apparatus and it may either be caused by 1) a direct action, 2) a stimulating substance liberated by adrenalin, or 3) a sensitizing action to the stimulating substance constantly being liberated by local ganglia but normally rapidly destroyed.

It cannot be a direct action, because adrenalin produces only inhibition on the completely nicotized gut and gut cooled for a longer period (73 hr.).

The fact that no stimulation was found with either a small dose or after washing away a big dose of adrenalin, following atropine and the abolition of stimulation when atropine followed it, is highly suggestive. Atropine even abolished the stimulation caused by a large dose of adrenalin following eserine or acetylcholine as has been previously reported (13, 6). In addition, the report of the liberation of an acetylcholine-like substance from the heart of frog by adrenalin (14), the liberation of acetylcholine in the blood by adrenalin (15), and the increased synthesis of acetylcholine to the extent of 50 to 150 per cent by a minute dose of adrenalin,  $10^{-8}$  to  $10^{-5}$ , (16) adds further support. Moreover, the literature also reports (17, 18) formation of acetylcholine in the fresh gut or when cooled only for a short period. The lack of its formation by the embryonic guinea pig gut at a period when there was no development of nervous structure in it has also been reported.

All of these observations indicate that the substance responsible for the stimulation is something like acetylcholine liberated from the local nerve elements. It is noteworthy that the rabbit's gut has been found sensitive to acetylcholine in a concentration of 1 in  $30 \times 10^{-13}$ .

The recovery and overaction after adrenalin further substantiates the evidence already reported for the similar liberation of acetylcholine (19-22). It has been called an 'atypical action' and a 'drug-fading phenomenon.' These experiments

prove that neither recovery nor overaction is caused by destruction of adrenalin in the bath within the experimental time.

#### SUMMARY

Adrenalin exerts a double action on the gut: stimulation takes place even by an inhibitory dose when preceded by eserine or acetylcholine. This stimulation is abolished by atropine. The stimulation always took place in the fresh gut and never when cooled or nicotinized. The presence of parasympathetic tone seems to be a necessary condition. The gut showed recovery and sometimes overaction after an inhibitory dose of adrenalin, although the gut remained in contact with adrenalin in the bath. Stimulation, recovery and overaction appear to be caused by the liberation by adrenalin of acetylcholine from the nerve elements present in an isolated piece of the gut.

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# CREATINE-CREATININE INDICES OF DIABETIC SUBJECTS AND EFFECT OF MUSCULAR DEGENERATION

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PRIOR to the discovery and application of insulin, the hormone now used to control diabetes, some studies were reported on the excretion of creatine-creatinine and the collateral effect of diets upon these excretions during the course of this disease. Bürger and Nachwitz (1) found that on a carbohydrate-free diet, considerable amounts of sugar were excreted and similarly on a creatine-free diet, there ensued some excretion of creatine. Copious creatinuria occurred in diabetics without appearing to increase the total creatinine excretion. While severe cases of diabetes, exhibiting excretion of acetone substances, continued to excrete creatine independently of the food, the quantity of this latter excretion was influenced by the consumption of meat. However, mild cases of diabetes behaved like normal individuals. Lampert (2) found an increase in the excretion of acetone bodies coincidental with creatinuria when carbohydrates were given. In the few cases of diabetes investigated, the creatinine output was considerably lower than normal. Lauritzen (3) found that restriction of carbohydrates led to creatinuria and he believed creatinuria was a valuable early sign of the precomatose state. It is apparent from these few early studies that the significance of creatine-creatinine excretion had neither been conclusively determined nor satisfactorily related to faulty metabolism.

Tsuji (4) noted, with an artificially induced glycosuria, an association of creatine excretion. He cast doubt upon the contention that such creatinuria was due to the presence of acetoacetic acid or other acetone substances. Lohmann (5) demonstrated that creatinuria was associated with a diminished utilization of sugar in rabbits. Reuter and Schlessmann (6) considered creatinuria a sign of loss of glycogen and deficient synthesis in the muscle. Wang (7) evaluated creatinuria produced under a variety of experimental and clinical conditions as an unspecific phenomenon.

There is evidence that insulin regulates the redistribution of tissue glycogen and glucose and possibly their metabolism (8). Whether this regulatory mechanism exerts some salutary influence upon other abnormal phenomena associated with diabetics, such as creatinuria, constitutes a subject worthy of thorough investigation. Should insulin be found ineffective as a corrective for creatinuria, then it follows that this disease process is more complicated than anticipated and we must search further for coordinate leads of consequence.

Caspe and Cameron (9) by a tissue culture technique showed that creatine plays a role in cellular proliferation. The function of creatine in muscular contraction and glycolysis has been dwelled upon by several investigators. Since muscular degeneration is sometimes a concurrent phenomenon observed in diabetes, the importance of creatine in this connection warrants investigation. Lazere, Thomson and Hines (10) found in the atrophy of rats' muscles following denervation or other causes a more rapid decrease in glycogen than in creatine content. Regeneration of the affected nerves brought about redistribution of muscle glycogen more rapidly than creatine. The fibrillary activity of these muscles was also studied.

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Caspe, Davidson and Truhlar (11) report creatinuria was found in all diabetics of both sexes taken at random. Since lethargy and a growing fatigue are some of the extrinsic clinical signs associated with the progress of this disease, it was decided to study the creatine and creatinine index of diabetics in connection with their muscular tone and fibrillary activity. The biochemical aberration in creatine metabolism is a factor perhaps of equal importance to the decrease in available glycogen in the tissues.

#### EXPERIMENTAL METHODS AND MATERIALS

In the diabetic clinic, blood sugar analysis is done routinely. During an extended study of 100 subjects with creatinuria, a decision was made to investigate intensively the urinary sugar, creatine and creatinine of 34 diabetic clinic subjects (22 females and 12 males) most of whom have been afflicted with this disease for a long time. The diabetes was controlled by means of insulin. The body weights of these subjects were maintained on a 1500 calorie diet (not creatine-free) consisting

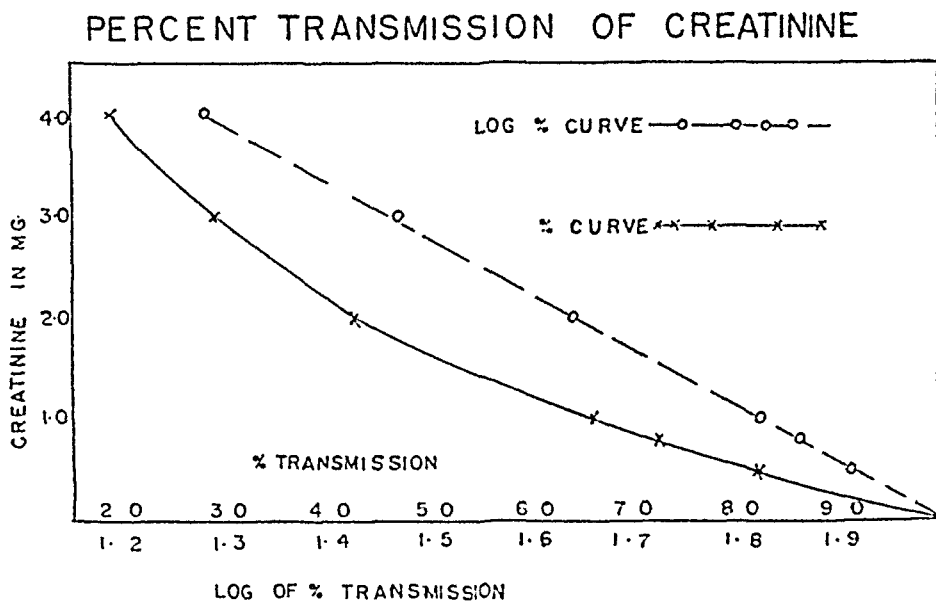


Fig. 1

of a daily ration of 150 grams carbohydrate, 60 grams protein and 70 grams fat. The urine of each patient was collected for 24 hours and a sample preserved with thymol was withdrawn for creatine and creatinine determination by a modification of the method of Folin (12). The urine was refrigerated and analyzed within 48 hours.

One ml. of a 24-hour specimen of urine was mixed with 20 ml. of a saturated solution of picric acid and autoclaved for 20 minutes at a 15-lb. pressure. The solution was then cooled in an ice bath for 15 minutes. After removal from the ice bath, 1.5 ml. of a 10 per cent NaOH solution was added and this mixture was allowed to stand 10 minutes after which it was diluted to a suitable volume. The samples were read within 10 minutes in a Lumetron photoelectric colorimeter at a 550  $\mu$  wavelength. The creatinine standards were dissolved in dilute HCl.

At the start duplicate determinations of creatinine standards were run and the curves show the average percentage transmission of light as well as the log of percentage transmission of light plotted against milligrams concentration of creatinine.

The concentration of creatinine in urine samples can be determined by reading these samples in the photoelectric colorimeter and ascertaining the creatinine values from the curves. However, standards were always run with each batch of urines as a precautionary measure to compensate for any manual variations. All samples including standards were run in duplicates and the deviation between duplicate readings was found to be less than 1 per cent while in most cases the deviation was insignificant. Using known standards of creatinine, we found the average recovery to be 97.5 per cent.

All the experimental urines were found to be acetone-free although many contained glucose. Control experiments were run to determine the possible interference of glucose with the accuracy of the creatine and creatinine determinations. Toward

TABLE 1. MALE DIABETICS

SUBJECT	WEIGHT	AGE	BLOOD SUGAR	URINE SUGAR	TOTAL CREATININE	CREATINE	CREATININE INDEX	CREATINE INDEX	MUSCLE TEST
	kg.		mg. %		mg/day	mg/day			
B. K.	61	32	249	2.8%	1363	388	22	6.3	Neg.
S. S.	64	36	150	Neg.	1800	500	28.1	7.8	Neg.
D. S.	67	43	95	Neg.	2328	823	35	12.3	Neg.
H. A.	68	45	121	Neg.	1013	200	15	2.9	Neg.
R. S.	70	47	180	F. T.	1900	440	27	6.3	Neg.
H. A.	69	48	194	F. T.	2048	700	30	10.1	Neg.
S. C.	68	51	166	F. T.	1870	587	26	8.5	Neg.
G. G.	59	53	128	F. T.	1785	368	30	6.2	Neg.
W. P.	66	53	336	3.0%	4655	700	75	10.6	++++
W. P. <sup>1</sup>	66	53	280	2.2%	5280	1590	93	24.1	++++
A. K.	55	53	140	F. T.	2795	1081	51	19.6	++++
M. F.	60	59	155	Neg.	1620	500	27	8.3	++++
M. F. <sup>1</sup>	62	59	257	2.7%	2288	970	36.8	15.6	+++
M. F. <sup>1</sup>	61	59	—	Neg.	2538	586	41	9.6	+++
C. S.	59	69	295	1.5%	2241	981	38	16.6	++++

F.T. = Faint trace. <sup>1</sup> Duplicate determination made on same subject one week later.

this end, 14 determinations in duplicate were made as follows: A known amount of glucose was added (making the final concentration equivalent to 2%) to 1) a known amount of creatinine, 2) normal urine and 3) picric acid. These controls were subjected to the procedure and autoclaving outlined above. No significant variations were observed in comparing 1) standard creatinine with the same creatinine plus glucose, 2) normal urine with the same urine plus glucose and 3) picric acid with picric acid plus glucose.

Thirty-two of the 34 patients were subjected to a muscle fibrillation test, and the longitudinal and lateral fibrillations were noted. A visual estimation was made of their intensity. This test is described in many standard textbooks of physiology and neurology. It consists of producing muscular tension by a hammer blow, finger squeezing or snapping of the relaxed biceps and gastrocnemius muscles. When muscles are degenerating, the response to tension can be observed in the uncoordinated

twitchings of bundles of muscle fibers. Grinker (13) states that tapping or squeezing of muscles may cause the appearance of a fibrillation. "The significance of fibrillary twitching is generally stated to be a process of slow degeneration."

Of the 34 subjects, 4 were in the 30-age group, 14 in the 40-age group, 13 in the 50-age group and 3 in the 60-age group. Of the 11 subjects showing muscular fibrillary activity, 3 were in the 40-age group, 6 in the 50-age group and 2 in the 60-age group.

TABLE 2. FEMALE DIABETICS

SUBJECT	WEIGHT	AGE	BLOOD SUGAR	URINE SUGAR	TOTAL CREATININE	CREATINE	CREATININE INDEX	CREATINE INDEX	MUSCLE TEST
	kg.		mg. %		mg/day	mg/day			
S. R.	89	34	206	H. T.	2610	840	29	9.4	Neg.
S. C.	64	37	188	F. T.	960	230	15	4.6	Neg.
R. Z.	67	44	150	H. T.	1600	460	24	6.9	Neg.
E. S.	78	44	222	1.5%	2655	780	34	10.0	+
S. P.	54	45	168	H. T.	2745	810	56	15	+
S. P. <sup>1</sup>	54	45	139	F. T.	2700	1060	50	19.6	+
B. B.	63	46	180	H. T.	3280	1060	52	16.8	
G. F.	79	47	252	H. T.	2460	560	31	7.1	Neg.
V. H.	73	47	374	3%	1960	94	27	1.3	Neg.
F. I.	78.6	48	230	Neg.	1250	200	15.9	2.5	Neg.
F. I. <sup>1</sup>	77.0	48	262	Trace	1238	370	16.	4.8	Neg.
M. M.	55	48	200	H. T.	2398	1015	43.5	18	
E. S.	73	48	285	1.7%	3000	1420	41	19.5	++
E. F.	73	48	280	3.1%	1900	1238	26	16.9	Neg.
H. Z.	64	50	180	F. T.	1663	650	26	10.1	Neg.
F. G.	89	50	300	3.8%	1170	180	13	2	Neg.
C. K.	88	50	490	4.2%	2640	390	30	4.4	Neg.
A. K.	59	50	265	1.6%	2725	1208	46	20	+++
F. G.	88	51	225	H. T.	1690	670	19	7.6	Neg.
A. H.	70	51	185	Neg.	1977	945	28	13.5	Neg.
S. K.	55	52	260	F. T.	2260	1080	41	19.7	++
S. K. <sup>1</sup>	55	52	284	H. T.	2740	1120	50	20.4	++
R. W.	72	52		4.8%	2676	894	37.2	12.4	++
A. B.	57	60	339	7.1%	1845	489	32.3	8.6	+++
A. B. <sup>1</sup>	57	60	—	4.0%	4292	2324	75.	40.	+++
S. G.	66	67	203	Neg.	1181	500	18	7.6	Neg.

F.T. = Faint trace. H.T. = High trace. <sup>1</sup>Duplicate determination made on same subject one week later.

In tables 1 and 2 (males and females respectively) the various analyses and the creatine-creatinine indices are given for each subject. These indices are obtained by calculating the milligrams of creatine and creatinine excreted in 24 hr/kg. of body weight. The subjects are tabulated in the ascending order of their ages and any particular age group can easily be identified and studied.

Table 3 is a tabulation of the creatine-creatinine indices of 7 normal subjects (4 males and 3 females) free from any disease and representing selections from various age groups. These figures serve as controls for the creatine-creatinine de-

terminations of the diabetic group. Table 4 is a recapitulation of creatine-creatinine average indices compiled from the data given in tables 1, 2 and 3.

## DISCUSSION

There were 41 creatine-creatinine determinations made on the 34 diabetic test subjects. *M. F.* (in table 1) is the only subject with a positive muscle test who showed a creatinine index of 27. However, two subsequent determinations on *M. F.* resulted in creatinine indices of approximately 40.

TABLE 3. CONTROLS

SUBJECT	SEX	WEIGHT	AGE	TOTAL CREATININE	CREATINE	CREATININE INDEX	CREATINE INDEX
		kg.		mg/day	mg/day		
<i>M. C.</i>	M	84	25	2122	20.4	25.2	0.24
<i>J. T.</i>	M	77.3	30	2163		27.9	0
<i>J. T.</i>	M	79.5	35	2189	22	27.5	0.28
<i>S. C.</i>	M	79	43	1830	60	23.2	0.75
<i>M. C.</i>	F	64	31	1739	57	26.8	0.89
<i>H. T.</i>	F	70.5	35	2001	20.4	28.4	0.29
<i>T. W.</i>	F	72.7	57	1833	61.5	25.2	0.84

TABLE 4. RECAPITULATION

	NO. SUBJECT DETERMINATIONS	TOTAL CREATININE INDEX	AV. CREATINE INDEX	RATIO CREATINE: CREATININE
CONTROLS.....	7	26.3	0.47	.018
Diabetics with creatinine indices: males, below 30; females, below 25.....	13	20.5	5.8	.282
Diabetics with creatinine indices: all below 30.....	18	22.3	7.0	.314
Diabetics with creatinine indices: all above 30.....	23	45.6	15.5	.340
Diabetics with positive muscle test and above 30 creatinine index.....	16	49.8	17.6	.353

Sixteen of the 23 subject determinations with an above 30 creatinine index exhibited this high index associated with muscle fibrillary activity; 2 subjects with similar high index were not tested for muscle fibrillary activity and the 5 remaining subjects with a negative muscle test had the following creatinine indices: 30, 30, 35, 30 and 31.

There does not seem to be a direct correlation between muscle tone and the severity of diabetes as manifested by a high blood and urine sugar, but there seems to be some connection between the age of the subject, the duration of his disease and his muscular condition.

Comparing the recapitulation figure (table 4), it is obvious the above 30 index average for creatinine is double the below 30 index average. The average creatinine index of those with a fibrillary muscle activity and an above 30 index is more than

double the creatinine average of those with a below 30 index. The average creatine index in a similar comparison is increased two and one-half fold.

The average creatinine index of diabetics with a positive muscle test increased over the comparable control average index by the factor 1.9 whereas the creatine index in a similar comparison increased by a factor 37.4 which appears to be significant. A comparison of the creatine-creatinine of those diabetics who exhibit a positive muscle test with the controls shows that this ratio has increased by a factor 19.8 (See table 4).

The normal excretion of total creatinine in 7 young adult males on various types of diet can be found in the recent studies of Friedemann *et al.* (14).

Maranon, Collazo and Almela (15) found by analysis of the deltoid muscle tissue of normal men and severe diabetics an average reduction of better than 20 per cent in the phosphagen content of diabetics in comparison with normals. The reduction in more active muscles may be even greater. This condition must reflect the effect of prolonged creatinuria. Glycosuria and creatinuria are related to the reduction of these components in the tissue. Muscle metabolism is affected by the reduction in its glycogen and creatine content which contributes to the muscular and possibly neuromuscular damage as noted by our reported fibrillation tests.

#### SUMMARY

Forty-one urinary creatine-creatinine determinations of 34 diabetic clinic subjects show that these excretions increase as compared with normal controls. Those diabetics with fibrillary muscular activity manifest an average creatinine index which is more than that of the normal controls while their average creatine index is more than 37 times that of the normal controls. This marked increase in creatine excretion appears to be a part of the diabetic syndrome. The extent of this increase seems to correspond to a condition of muscular degeneration.

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# CHEMICAL MEDIATORS AS PROMOTING AGENTS OF THE ORIGIN OF HEART RHYTHM<sup>1,2</sup>

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**D**EMOOR and Rijlant (1, 2) demonstrated that both aqueous and alcoholic extracts of nodal tissue (Keith-Flack nodes, Aschoff-Tawara centers, His' fasciculus, Purkinje's net) are capable of inducing regular rhythm in a heart fragment in which only rare twitchings, with uneven intensity and frequency, were evident. The 'wild activity' of the myocardium is changed into 'rhythmical automatism' under the influence of the nodal tissue extract. The extracts of nodal tissue when heated to 60° C. lose their rhythm-inducing capacity.

Haberlandt (3) states that in Batrachia a 'Herzhormon' exists and Zwaardemaker (4) considered that the active substance of the heart rhythm is 'automatine' formed by the action of potassium and other radioactive bodies, on 'automatinogène' existing throughout the body and conveyed by the blood into the heart, where it accumulates chiefly in the nodal tissues; the essential rhythm-inducing rôle of nodal tissue extracts has been confirmed by others (5-13).

Bacq and his co-workers (14), however, are not in agreement with this concept. It is possible to induce rhythm in the left auricle by other means. Unbalanced salt solutions can provoke (even in the non-smooth muscle) a series of contractions more or less regularly rhythmic. In the left auricle's muscle, Baryum chloride (Kruta), good oxygenation (Brouha and Bacq), histamine (Rigler and Tiemann), repeated induced electric shocks at regular intervals (Fredericq), and probably any other technics can lead the left auricle to contractions that simulate normal regular rhythm.

With a view to determining the reciprocal relations between the nodal tissue's rhythm-inducing capacity and the chemical mediators of the cardiac nerves, the nodal tissue—acetylcholine and adrenaline inter-relations—in rhythm-inducing capacity, the experiments described below were made with the left auricles of rabbits.

## METHOD AND RESULTS

Immediately after killing the rabbit by a blow on the head, the heart was exposed through an opening in the chest; after ligature of the vena cava, the heart was removed and washed in tepid Locke's solution. The auricles were carefully separated from the ventricles. The isolation of the left from the right auricle was accomplished as precisely as possible. The separation should be done in such a way as to totally deprive the left auricle of nodal tissue (left extremity of Keith-Flack nodes, inter-auricular septum, several parts of the nodal 'Tawarien' system).

The left auricle thus prepared was widely opened and tied on one side to a myograph lever and on the other to the terminal apparatus hook. The preparation

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was immersed in 50 cc. of Locke's solution maintained at  $38^{\circ}\text{C}$ ., through which oxygen was continuously bubbled. The nodal tissue aqueous extract employed was prepared from sheep's heart after the method of Demoor and Rijlant (1, 2).



FIG. 1. RABBIT'S LEFT AURICLE IMMERSED IN LOCKE'S SERUM. Auricle prepared at 2 hr. 20 min.; first twitching at 3 hr. 30 min.; 4 hr. 55 min., addition of 5 cc. of aqueous extracts of Keith-Flack nodes, inactivated through heat ( $60^{\circ}\text{C}$ .) during half-hour. Ulterior addition *in vivo* of associated adrenalin (5 drops of solution at 1/1000), acetylcholine (2 drops of solution at 1/10,000); 5 hr. 19 min., rhythmical reaction, continuing until 5 hr. 56 min.; 6 hr. 25 min., addition of associated adrenalin (3 drops), acetylcholine (2 drops); 6 hr. 28 min., rhythmical reaction continuing until 7 hr. 37 min.

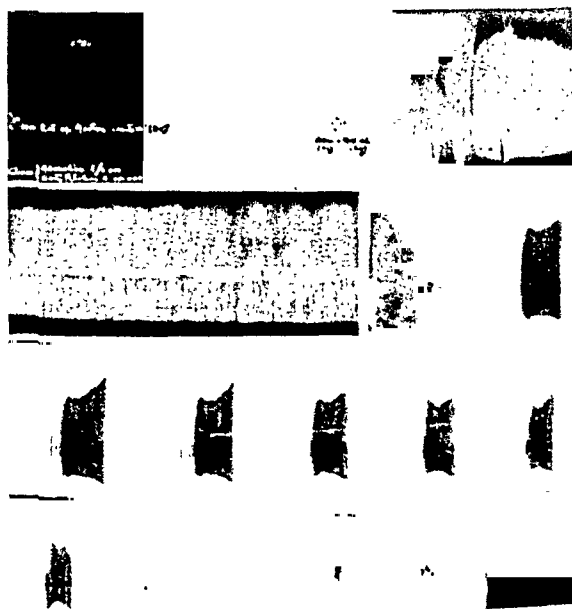


FIG. 2. RABBIT'S LEFT AURICLE IMMERSED IN LOCKE'S SERUM. Auricle prepared at 3 hr. 25 min.; first twitching at 5 hr. 20 min.; 6 hr. 10 min., addition of 10 cc. of aqueous extracts of the nodal tissue inactivated through heat ( $60^{\circ}\text{C}$ .) during half-hour; 6 hr. 16 min., addition of associate adrenalin (5 drops), acetylcholine (2 drops). A few minutes later, rhythmical reaction, until 6 hr. 45 min. (continuous); 6 hr. 45 min.-7 hr. 25 min., continuation of the rhythmical reaction intermittently.

*Effect of Nodal Tissue Extracts on Left Auricle Activity.* Addition of nodal extracts can induce rhythm-automatism of the left auricle and make it sensible to adrenalin and acetylcholine, as has been demonstrated by Demoor and Rijlant. The present observations have shown that the association of adrenalin (5 drops of solution of 1/1000) and acetylcholine (2 drops of solution at 1/10,000) has better and longer

lasting effect than that of the same dose of adrenalin alone. Once the rhythm-automatism induced by the nodal tissue extracts ceases, it can be made to reappear by the addition of adrenalin plus acetylcholine much easier than through the addition of adrenalin alone. Similarly, when the rhythmic activity induced by the nodal tissue extracts has almost ceased, the rhythm of the contractions is no longer even, but occurs in groups; adrenalin alone is capable only of increasing the amplitude of each group of contractions, but the inclusion of acetylcholine causes the recovery of an even rhythm.

If the nodal extract is heated for 30 minutes to 60° C. on a water bath, it will no longer be capable of inducing rhythm in the left auricle. However, this extract is still capable of making the left auricle sensible to the action of adrenalin. The addition of associated adrenalin plus acetylcholine to the left auricle, properly isolated, produces no rhythm reaction whatever.

*Left Auricle Activity When Isolated, Submitted to the Nodal Heated Extract's Action, Reactivated by Adrenalin and Acetylcholine.* If to the nodal extract inactivated through heat, associated adrenalin and acetylcholine are added, the extract recovers its rhythm-inducing properties. These properties do not reappear through the addition of adrenalin alone.

When the left auricle is submitted to inactivated extract, and later associated adrenalin-acetylcholine is added, it recovers the rhythm-inducing capacity. This rhythmical reaction is sometimes almost immediate, lasting and constant. On the contrary, the rhythmic action obtained through adrenalin alone is always late, less lasting and less constant. The rhythmic reaction appears to be always a function of the adrenalin/acetylcholine concentrations, when added to inactivated extract. In brief, the nodal extracts inactivated through heat recover their rhythm-inducing properties by the addition of a combination of adrenalin-acetylcholine. The latter permits inactivated extract to provoke reactions similar to those caused by non-inactivated nodal extract.

These facts lead to the assumption that in the 'inactivated' extracts a substance exists (not self-rhythm-inducing), which may be called 'pre-rhythmia', and this substance under the action of acetylcholine and adrenalin becomes a rhythm-inducing substance, which may be termed 'rhythmia'. On the other hand, and according to Loewi's theory of the chemical mediators today universally accepted, the nerves as they are known act through chemical substances released at their extremities, acetylcholine and adrenalin. In the body, acetylcholine in the presence of the sympathetic substance (adrenalin) might act upon the 'pre-rhythmia' (main characteristic substance of the nodal tissue), which might transform it into a rhythm-inducing substance. Moreover, the frequency of the heart rhythm would depend at all times on the relation to the acetylcholine-adrenalin, in action.

#### SUMMARY

Extracts of nodal tissue from sheep's hearts change the 'wild activity' of the myocardium of the rabbit into rhythmic activity. Such extracts of nodal tissue when heated to 60° C. lose their rhythm-inducing capacity. Acetylcholine, in the presence of adrenalin (an association not rhythm-inducing by itself) acting upon extracts

inactivated by heat (not rhythm-inducing by themselves) restores the rhythm-inducing property to these extracts.

These facts are interpreted as indicating that acetylcholine in the presence of adrenalin acts upon a substance called 'pre-rhythmica' transforming it into a rhythm-inducing substance called 'rhythmica'.

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# INFLUENCE OF ACUTE EPINEPHRINE HYPERTENSION ON CALCULATED RESISTANCE OF CANINE FEMORAL VASCULAR BED

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**F**LOWMETERS such as the rotameter or bubblemeter when used to measure arterial inflow delay the arrival of intravenously injected substances in the periphery by the time necessary to displace the blood in the flowmeter system. There is therefore a short period of time during which the measured blood flow is under the influences of only the central vascular actions of the substance. When the femoral arterial flow of anesthetized dogs is measured by one of these meters, it will be found that during the acute pressor response to intravenous epinephrine the flow will increase at a faster rate than the pressure. This will result in a decrease in the calculated resistance ( $P/F$ ). As soon as the epinephrine has passed through the metering system and reached the femoral vascular bed there is an immediate decrease in flow and an increase in resistance due to local vasoconstriction.

This paper is concerned with an analysis of the factors which bring about this fall in calculated resistance during the time that the epinephrine is acting only central to the femoral vascular bed.

## GENERAL METHODS

Dogs of either sex, weighing from 5 to 20 kg., were anesthetized with pentobarbital sodium (10-20 mg/kg.) administered intravenously about 30 minutes following a subcutaneous dose of morphine sulfate (10 mg/kg.). Arterial blood flow was measured with a Shipley optically recording rotameter (1). Arterial pressure was recorded with either a Hamilton manometer (2) or a strain-gauge recording system<sup>1</sup>. Heparin<sup>2</sup> was used as the anticoagulant and most of the dogs were atropinized (0.5 mg/kg.). Epinephrine (0.005 mg/kg.) was injected intravenously.

The peripheral resistance was calculated by the simple formula  $P/F$  in which  $P$  is the mean arterial pressure in mm. Hg, and  $F$  the blood flow in cc. per minute. The arterial pressure was recorded from a side-arm in the rotameter circuit, since, as will be pointed out in this paper, arterial pressure recorded directly from some other artery, the carotid for example, is not necessarily the true arterial perfusion pressure in the flowmeter system. The rotameter was calibrated in each dog

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<sup>1</sup> The multi-channel Statham-Heiland strain gauge recording system used in some of these experiments was constructed under a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

<sup>2</sup> Much of the heparin used in these studies was kindly supplied by Lederle Laboratories and The Upjohn Company.

using a graduated cylinder and stop-watch. The calibration points always included the highest and lowest flows encountered, together with several intermediate points.

As a check on the recording system the rotameter was used to determine the influence of pressure on the flow of water through a fixed resistance (glass tube). When the pressure was increased very suddenly the calculated resistance increased due to the inherent lag in the recording system. When the pressure was increased less rapidly, at a rate comparable to that produced *in vivo* by intravenous epinephrine,

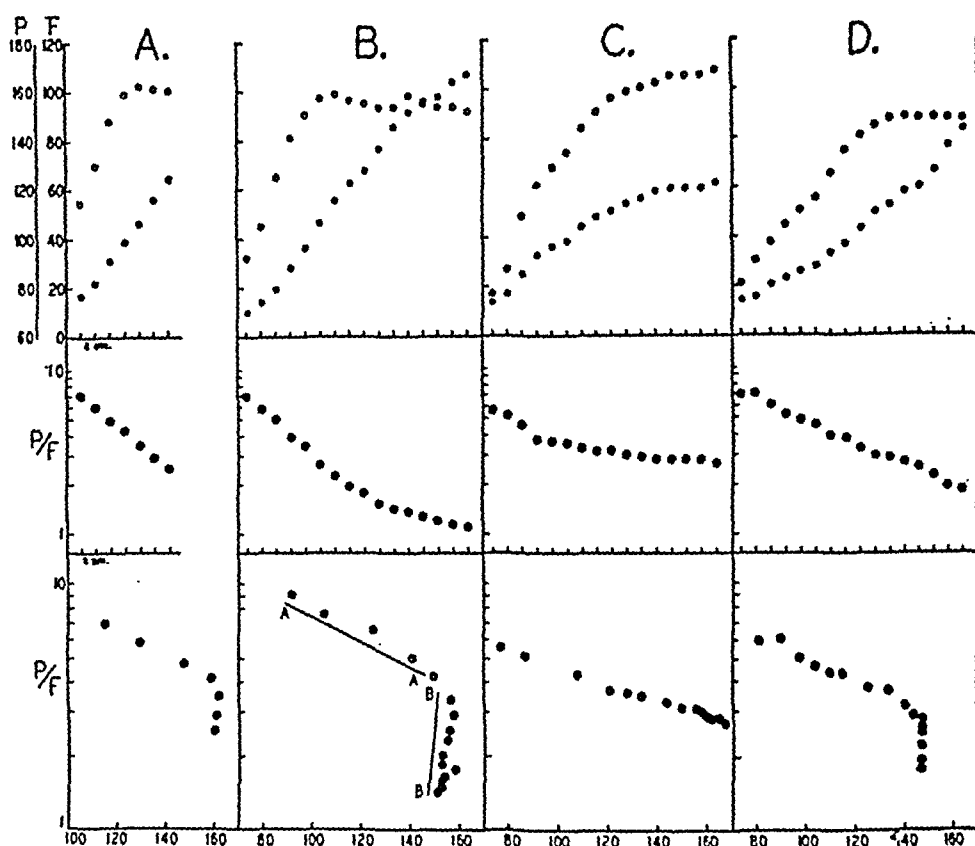


Fig. 1. PRESSURE-FLOW RELATIONSHIPS in femoral vascular bed during the acute hypertension induced by intravenous epinephrine. Records from above downward: Arterial perfusion pressure (open circles) in mm. Hg, arterial blood flow in cc/min., and  $P/F$  (see text) plotted against time (time marks at 2 sec. intervals). Bottom record:  $P/F$  plotted against arterial perfusion pressure. The  $P/F$  is plotted on a logarithmic scale for convenience only. A, mean results of 17 determinations using the ordinary rotameter arrangement. B, mean results of 6 determinations using the long tube (see text) to delay the direct local action of the epinephrine. C, mean results of 6 determinations using the long tube and recipient leg. D, result obtained by injecting epinephrine into the donor and recipient animals simultaneously.

the calculated resistance remained unchanged. These results indicated that the effects to be described were not recording artifacts.

## RESULTS

The ordinary rotameter arrangement contained about five cc. of blood within the meter and connecting tubes. This volume of blood delayed the peripheral effect of epinephrine for 10 to 12 seconds. Figure 1A illustrates the results obtained using this arrangement.

In order to delay further the direct peripheral effect of epinephrine and increase the observation period a plastic tube about twelve feet long was placed between the rotameter and its blood supply. This tube had a volume of about 50 cc. and delayed the peripheral arrival of the epinephrine 30 to 40 seconds. To prevent cooling of the blood the tube was coiled within the abdominal cavity of the dog. The results obtained with this arrangement are shown in figure 1B.

Two distinct phases in the falling resistance were apparent: the decrease during the rise in pressure (line A-A in the figure) and the continuing decrease when the pressure was no longer rising (line B-B). In an attempt to separate these phases cross-perfusion studies were done. Arterial blood from a donor dog was led through the long tube and rotameter into the femoral artery of a recipient dog. Femoral venous blood from the recipient's perfused leg was returned to the donor through another rotameter. The recipient leg was ligated tightly central to the cannulae to prevent interchange of blood between the two animals. The adequacy of the ligature was demonstrated by the fact that epinephrine administered intravenously to either animal had no effect on the arterial pressure of the other and that atropine given to the donor did not atropinize the recipient. The recipient leg however, was atropinized as shown by the absence of vasodilator response to an intra-arterial injection of 0.02 mg. of acetylcholine.

The results obtained on the administration of epinephrine to the donor are shown in figure 1C. It will be seen that the second phase in the decrease in resistance was eliminated. This suggested that this phase was due to neurogenic factors resulting from the increase in arterial pressure.

Epinephrine administered intravenously to the recipient dog only produced a marked increase in flow through the perfused leg with no change in the perfusion pressure from the donor animal. Epinephrine was then administered simultaneously to both donor and recipient with the results shown in figure 1D. It will be seen that the second phase of the decreased resistance was present.

To demonstrate that the nerve supply to the perfused leg was intact, and to attempt to block the neurogenic vasodilation, tetraethylammonium bromide (5 mg/kg.) was administered intravenously to the recipient animal. An immediate increase in blood flow in the perfused leg occurred. When epinephrine was now administered to both animals simultaneously the second phase in the decreasing resistance was no longer present as shown in figure 2.

#### DISCUSSION

The decrease in calculated resistance in the femoral vascular bed during the acute pressor response to epinephrine is due to at least two distinct mechanisms. The first of these is related directly to the increasing pressure and is probably identical to that described by Green *et al.* (3). These authors showed that as perfusion pressure increases the calculated resistance decreases and demonstrated that this phenomenon is probably due to vascular distention rather than to changes in apparent viscosity of the blood. The existence of vascular distention was easily apparent in our cross-perfusion experiments. Here, the rate of increase in arterial inflow was always greater than the rate of increase in venous outflow. During any

one response to epinephrine at least five cc. of blood entered the leg which was not immediately accounted for in the venous outflow.

In the presence of maximal dilation (produced by the intra-arterial administration of 20 mg. of sodium cyanide to the perfused recipient leg) the effect of vascular distention is eliminated as shown in figure 2. In this case the vascular bed behaves like a rigid tube and during the increase in pressure the  $P/F$  relationship becomes linear. This finding is in accord with the results of Whittaker and Winton (4) who used a perfusion method in which the vascular bed was dilated.

The second mechanism responsible for the decreasing resistance is reflex vasodilation. This effect does not occur in the denervated vascular bed (the perfused recipient leg) unless the arterial pressure of the recipient dog is elevated. There is

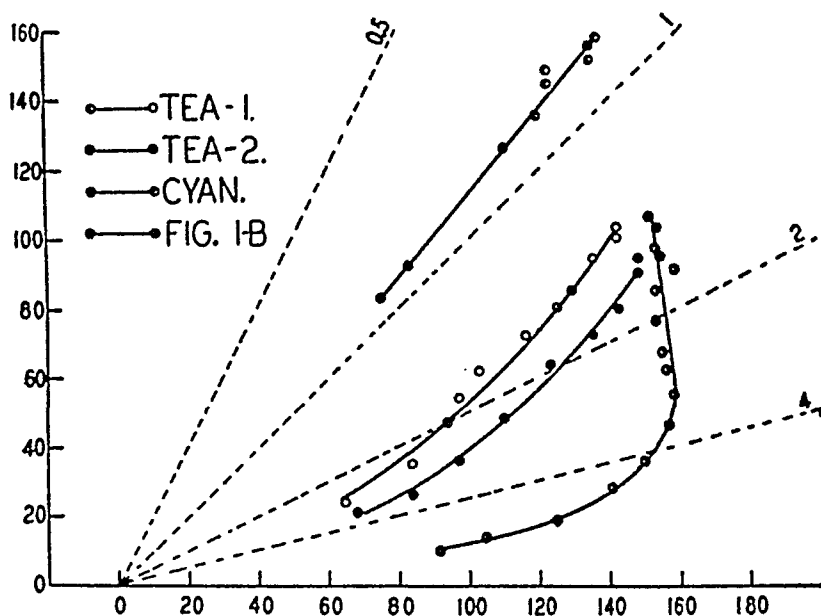


Fig. 2. PRESSURE-FLOW RELATIONSHIPS in femoral vascular bed. Ordinate: Blood flow in cc/min. Abscissa: Arterial pressure in mm. Hg. The broken lines indicate the slopes of calculated resistances ( $P/F$ ) of 0.5, 1, 2, and 4. TEA-1, epinephrine to donor animal only following TEA to recipient animal. TEA-2, epinephrine to both animals following TEA to recipient. CYAN., epinephrine to donor following administration of sodium cyanide to recipient leg. FIG. 1B plotted for reference.

no reason to doubt that this is a demonstration of the classical concept of reflex dilation mediated through the pressure receptors of the aorta and carotid sinus. The dilation is due to a decrease in sympathetic vasomotor tone since the atropinized leg cannot respond to cholinergic impulses, and since there is no evidence for the existence of adrenergic vasodilator fibers in the dog's leg (5).

Although the marked decrease in calculated resistance due to elevated pressure as described in this paper is apparent only because of the recording method used, the mechanisms responsible for it—vascular distention and reflex dilation—are always operative. This can be shown by the difference in peripheral constriction produced by intra-arterial and intravenous epinephrine. Only a small intra-arterial dose of epinephrine (0.001 mg. total) is necessary to bring about complete cessation of blood flow in the dog's leg. On the other hand, even large intravenous doses (0.005 to

0.05 mg. per kg.) do not produce cessation. In the latter case, after the direct peripheral action of epinephrine is established, the blood flow is seldom below the control value, although the calculated resistance is high because of the elevated pressure.

The reflex vasodilation encountered in this study was often of such a magnitude that a marked pressure drop occurred in the rotameter set-up if the intake cannula was of inadequate size. This resulted in a marked difference in the pressure recorded from the rotameter and that recorded from some other artery directly. It is apparent, therefore, that pressure recorded from the rotameter is not necessarily the true arterial pressure, and conversely, pressure recorded directly from an artery is not necessarily the perfusion pressure in the rotameter.

Although only epinephrine was used as the pressor agent in this study the same results have been obtained with other agents which can rapidly elevate the arterial pressure. These include arterenol, cobefrine, ephedrine, phenylephrine, privine and pituitrin.

#### SUMMARY

The calculated peripheral resistance ( $P/F$ ) of the canine femoral vascular bed is decreased during the acute pressor action of intravenous epinephrine *before* the direct action of epinephrine occurs.

The decrease in resistance is due to vascular distention and neurogenic reflex vasodilation resulting from the increased arterial pressure.

The influence of the decreased resistance on the direct local effect of epinephrine, and on the recorded arterial pressure is discussed.

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# RELATION OF INTRAVENTRICULAR DIELECTRICS TO THE UNIPOLAR LEADS<sup>1</sup>

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WHEN dielectrics as air, CO<sub>2</sub>, mineral oil etc. are placed in the right ventricle the initial ventricular deflection in direct leads from the anterior right ventricular surface are diminished, the normal RS complex being converted rapidly into a QS in most instances during the time that the dielectric is in the ventricle (1). The use of such agents could be a valuable tool for the study of the EKG. Of such dielectric substances, CO<sub>2</sub> is most useful since recovery from its repeated use is the rule while the rest of the materials mentioned may cause the death of animals into which they are introduced. The careful mapping of the proximal and distal areas of the anterior surfaces of the dog's heart (2, 3) in relation to unipolar leads immediately suggested that CO<sub>2</sub> might be used to lend support to or detract from the 'interference theory' of the production of the electrocardiogram.

Since the gas (CO<sub>2</sub>) reaches only the right ventricle the possibility existed that it would selectively eliminate the contribution of either the proximal or distal zones of any particular unipolar limb lead under investigation. Thus the unipolar EKG should be altered in a predictable manner. Furthermore, in the open chest, it is possible to change the position of the heart at will. By doing so, the anterior surface of the right ventricle could be brought into or out of either proximal or distal areas as desired and hence influence the EKG in a foreseeable way when this chamber is filled with carbon dioxide.

## METHODS

Mongrel dogs of 5 to 15 kg. were used. Anesthesia was obtained by intravenous sodium pentothal (20 mg/kg) and sodium barbital (150 mg/kg.). Electrocardiograms were obtained by use of three Cambridge Simplitrols where simultaneous leads were used. In other cases one or two machines were utilized as indicated. Carbon dioxide gas was introduced via a femoral or jugular vein, best through a cannula tied in the vessel.

Comparisons of simultaneous unipolar and standard limb leads were made with a normal intact chest or a thorax opened by a sternum-splitting incision. Shifts in the position of the heart were obtained by tilting the animal toward the left or right (open chest) from its starting supine position, or, better, by packing the heart into its new position with cotton but allowing the animal to remain supine. Direct leads were obtained via a saline wick electrode, placed with minimal pressure on the visceral epicardium.

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To secure forced mechanical extrasystoles a sharp needle was touched to the desired area. The point stimulated was usually indicated by a small hemorrhagic point. This point was useful in that the same area could be restimulated at will, allowing no doubt that identical points were used.

## RESULTS

*Effect of Carbon Dioxide in Right Ventricle on Simultaneous Pairs of Unipolar Limb Leads.* Animals remained supine in these experiments. Sometimes a standard limb lead was run along with the pair of unipolar leads. This served as an additional check since it is possible to compute the standard lead from the two related unipolar leads (3). These were both closed and open-chest experiments.

Fifty to one hundred cc. of CO<sub>2</sub> were given by vein while the electrocardiographs were running. Arrival of the gas at the heart was indicated by a 'mill-wheel' murmur. Continuous records were taken for several minutes after the murmur disappeared. VR and VL showed a decreased downstroke and an increased upstroke when compared to controls. In VL the R complex was often 'M' shaped and small, but the first movement of the beam was always upward. Effects in VR were usually more marked than with VL. The results in these two leads indicate a reduced proximal zone effect with an increased distal zone effect. VF changes were regularly a decreased upward movement of the beam and an increased downward deflection. This is the effect obtained when the proximal zone becomes more important and the distal zone less so.

When all three zones are considered together it becomes apparent that the areas affected when carbon dioxide enters the right ventricle are PPD according to the scheme of Nahum, Chernoff and Kaufman (3) (where each capital letter of the three given refers to proximal or distal area of VR, VL, and VF respectively). These effects are seen in figures 1 and 2.

*Effects of Carbon Dioxide in Right Ventricle on Unipolar Leads When Position of Heart Is Shifted Within the Open Chest.* If the boundary between proximal and distal zones could be localized on the anterior surface of the right ventricle then it should be possible to shift this border and hence the zones, in relation to the right ventricle, by changing the position of the heart within the chest. This boundary between proximal and distal zones was localized by use of forced mechanical epicardial extrasystoles in exploring the anterior surface of the right ventricle. As seen in figure 3, the boundary must exist somewhere between *points 1* and *2*. The first point gives an upward beam movement (distal zone) and the second point a downward deflection (proximal zone).

When the apex of the heart is moved to the animal's left (fig. 4, anterior aspect of heart) *point 2* has now entered the distal zone. This fact is demonstrated by the forced mechanical extrasystole from the same point which is now upright as compared to the control at the top or in figure 3. On the other hand, when the apex of the heart is moved to the animal's right, as in figure 5, *point 1* formerly in the distal zone has now become proximal. A forced mechanical extrasystole from *point 1* now first moves the beam downward (proximal zone) where it formerly moved upward (distal zone) as can be seen at the top or in figure 3.

While such effects can be shown in all three leads it is best demonstrated in VL

(figs. 4 and 5). This is so because the distal zones on the anterior surface for VL are small and roughly equal (2), being the right and left apical areas; consequently shifting the heart to the right makes the anterior right ventricle entirely proximal. Shifting the heart to the left brings the distal zone entirely on the anterior right ventricle.

When carbon dioxide enters the right ventricle with the apex moved to the right the downward deflection of the beam decreases (proximal effect) and upward deflec-

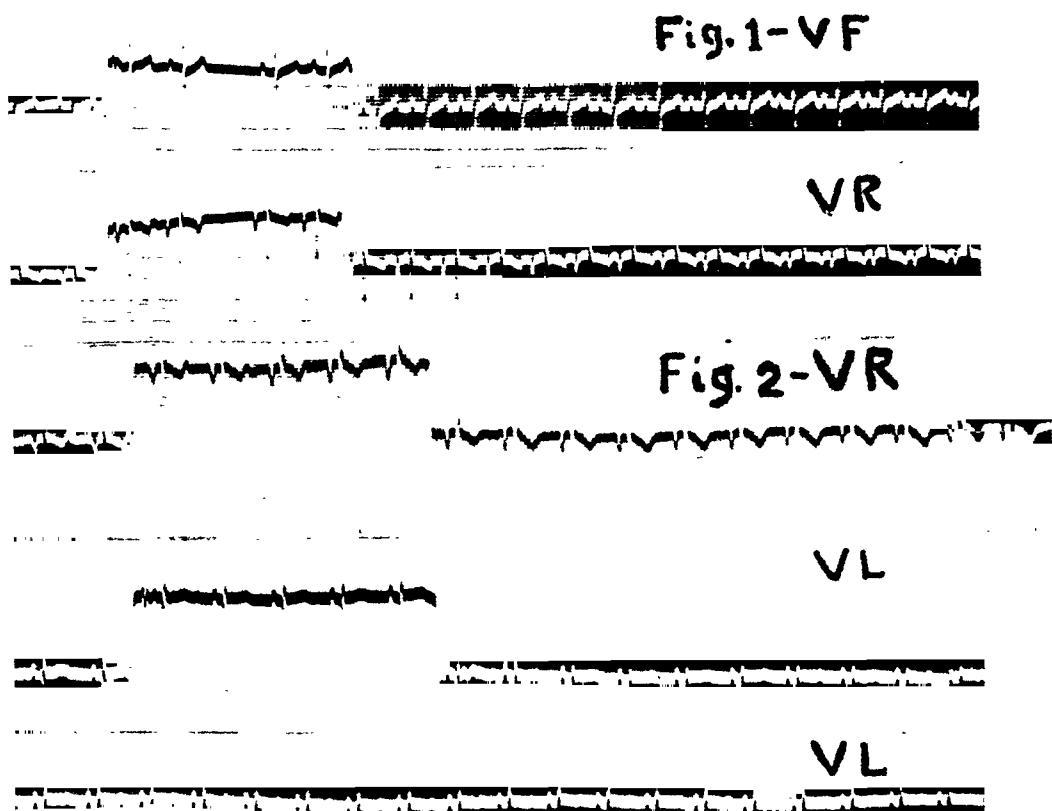


Fig. 1. FEMALE DOG, 5.6 kg. VF above, VR below. Two control beats at left. Millivolt signal indicates time during which 100 cc. CO<sub>2</sub> was injected into a femoral vein. Leads taken simultaneously.

Fig. 2. MALE DOG, 12.0 kg. VR above, VL middle and lower. Two control beats at left of upper and middle. Lower is a continuation of middle; effects develop more slowly in VL. Millivolt signal indicates time during which 100 cc. CO<sub>2</sub> was injected into a femoral vein. Leads taken simultaneously.

tions (distal effect) increase. This is shown in figure 6. Conversely, when the apex is placed more toward the left the downstroke (proximal effect) becomes larger and the upstroke, (distal effect) smaller. This is seen in figure 7.

VR and VF are less useful in this demonstration. VR has the anterior surface of the right ventricle entirely in the proximal zone with no distal zone representation (2). VF has the anterior right ventricle entirely in the distal zone (2). Hence, shifting of the proximal and distal zones by changes in anatomic position is much more difficult.

*Other Effects of CO<sub>2</sub> in the Right Ventricle.* All the standard leads show a decreased upstroke (R) and an increased downstroke (QS) with CO<sub>2</sub> in the right ventricle.

When carbon dioxide first enters the right ventricle extrasystoles often occur. These are, almost without exception in either VR or VF, in the same direction as forced mechanical epicardial extrasystoles obtained before or after, from the anterior surface of the right ventricle. In VR they are downward, in VF, upward (fig. 8). In VL the direction is variable and not always in agreement with forced extrasystoles. In one experiment simultaneous epicardial and endocardial leads, from points

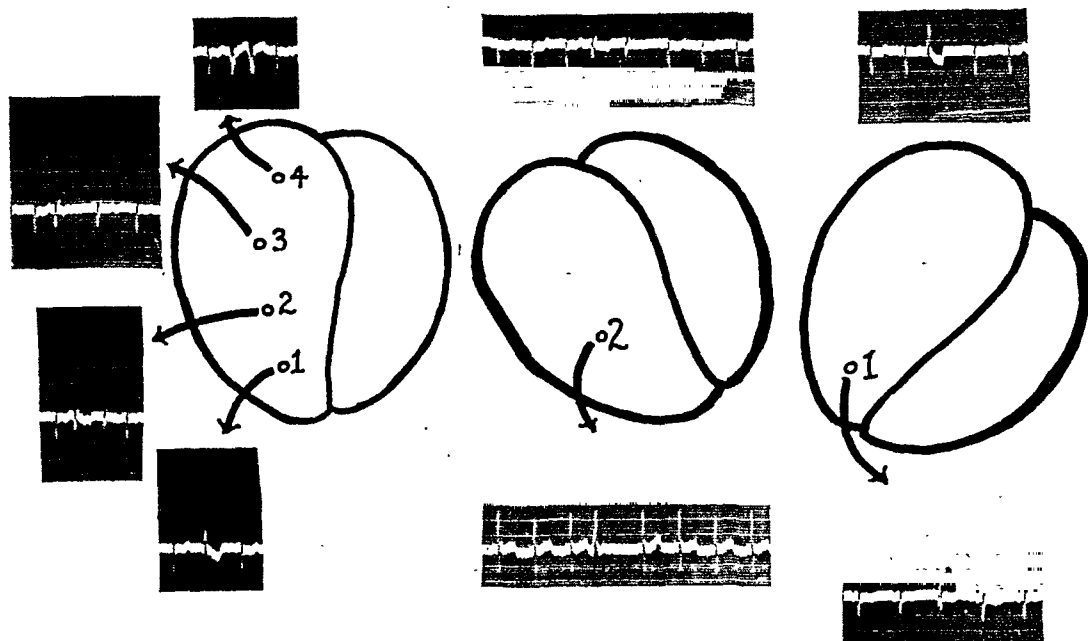


Fig 3 (left). MALE DOG, 11.1 kg. Dog supine, ventral view. VL throughout. Forced mechanical epicardial extrasystoles. 1) Distal zone, extrasystole bracketed by two normal complexes. 2) Proximal zone, 2 extrasystoles bracketed by 2 normal. 3, 4) Same as 2).

Fig. 4 (center; same as fig. 3). Above: Two CONTROL forced mechanical epicardial extrasystoles at point 2. Below: Forced mechanical epicardial extrasystoles at point 2 when apex of heart is rotated to animal's left about an anteroposterior axis at the base, with no rotation on its long axis (6). Three normal beats bracket the extrasystoles above; 3 normal precede and 4 normal follow the extrasystole below.

Fig. 5 (right; same as fig. 3). Above: FORCED MECHANICAL epicardial extrasystole at point 1. Below: Forced mechanical epicardial extrasystole at point 1 when apex of the heart is rotated to animal's right as in fig. 3. Both above and below, the mechanical extrasystole is bracketed by 2 normal complexes.

directly over one another, gave extrasystoles in the same direction during carbon dioxide in the right ventricle (fig. 9).

#### DISCUSSION

The effects of right intraventricular carbon dioxide are exerted principally against the anterior or ventral endocardial surface of that cavity in the supine position. Gas tends to come to the top. This has been shown to be so in venous gas embolism in patients (4) and animals (4) where turning the subject on the left side may be life saving. This maneuver floats the gas away from the pulmonary conus which it has been obstructing. Several animals in this series were saved in this manner when

the dose of carbon dioxide proved to be too large. When the gas impinges on the anterior endocardial surface it eliminates or diminishes the rôle of this part of the heart from the proximal or distal zone it occupies, depending upon the particular unipolar lead under observation. Proximal zone activity moves the beam downward; distal, upward (2, 3). The mechanism whereby the  $\text{CO}_2$  acts is a subject for subse-

Fig. 6



Fig. 7

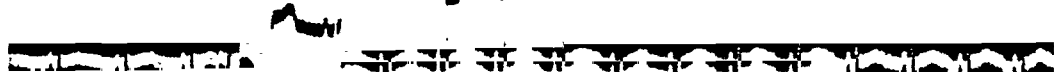
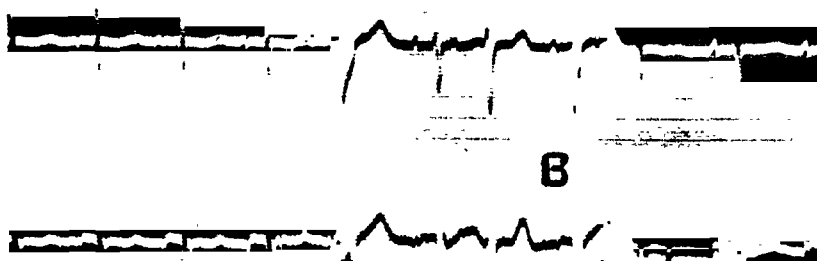


Fig. 8-VF

VR

Fig. 9 A



B

Fig. 6 (same as fig. 3). APEX TO RIGHT as in fig. 5. During millivolt signal 100 cc.  $\text{CO}_2$  injected into a femoral vein.

Fig. 7 (same as fig. 3). APEX TO LEFT as in fig. 4. 100 cc.  $\text{CO}_2$  was injected into a femoral vein, from after second control beat to end of millivolt signal.

Fig. 8. MALE DOG, 7.2 kg. Left: VF. Right: VR. Interruption of black bar in each case indicates duration of injection of 100 cc.  $\text{CO}_2$  into a femoral vein. These are simultaneously recorded.

Fig. 9. MALE DOG, 6.5 kg. A, Epicardial lead; B, endocardial lead. Camera running at double standard speed. Records simultaneous from points directly opposite one another. 100 cc.  $\text{CO}_2$  injected after second complex shown.

quent study and work now in progress. It is possible that  $\text{CO}_2$  on the endocardial surface produces a depressed area somewhat like KCl does on the epicardium. KCl sets up a neutral zone and permits recording of action currents from the other electrode (5). On the other hand it may be related entirely to the property of  $\text{CO}_2$  as a dielectric (1).

In the supine position VR has a proximal zone anterior which includes practically all the right ventricle (2). When gas is present this zone is eliminated and the distal zone predominates. As a result the downstroke decreases and the upstroke increases. In VF the right ventricle is almost entirely in the distal zone (2). Hence, elimination of this part of the distal zone decreases upward beam movements and accentuates the proximal zone effect of downward movements. With VL the proximal zone is the upper two thirds of the right ventricle and the distal zone is the lower one third of the same chamber (2). Therefore, carbon dioxide affects both zones but has a larger depressing effect on the proximal. Remaining distal zone effects predominate. Results are not so marked as in VR and VF but are ordinarily quite definite. The upward beam movements are often quite small and 'M' shaped. Thus it may be seen that all these results fit well with the 'interference' theory and the proximal and distal zones described (2, 3).

Changing the heart position fits equally well into a 'zonal interference' concept. It is known that in standard limb leads, after rotation of the heart on its antero-posterior axis at its base with practically no rotation on its long axis, the direction of the major initial deflection of electrically induced extrasystoles is often reversed in one or more leads (7). VL is most useful in this respect and the right ventricle may be made to include most or none of the distal zone by shifting the apex respectively to the animal's left or right. The difficulties which arise for the 'cavity potential' theory from the forced mechanical epicardial extrasystoles (used to outline the boundary between zones) are obvious. These oppositely directed extrasystoles are oriented toward the cavity in an identical manner. When the right ventricle includes most of the distal zone (apex to left) upward beam movements are decreased and proximal downward movements increased with CO<sub>2</sub>. The opposite is true when the right ventricle contains none of the distal zone (apex to right).

Extrasystoles induced by CO<sub>2</sub> are downward in VR because this is a proximal zone entirely. From VF the right ventricle is almost entirely distal, hence the extrasystoles are upward. VL is difficult to interpret because the right ventricle lies in both zones and stimulation may be proximal or distal from endocardial CO<sub>2</sub> effects. With VR and VF forced mechanical epicardial and CO<sub>2</sub>-induced endocardial extrasystoles have the same direction in any one lead under consideration. These findings fit well with a 'zonal interference' concept. Extrasystoles due to CO<sub>2</sub>-in lead VL may stimulate either (or both) proximal or distal zones while a single epicardial stimulation would be confined to one or the other zone. Correspondence of direction in extrasystoles from endo- and epicardium in every instance under such circumstances is not to be expected.

Leads directly from the epicardium with CV leads give an initial deflection which is upright (1). This then is a proximal zone effect which disappears with CO<sub>2</sub> in the right ventricle. If a unipolar lead explores from the right wrist, up the right arm to the right shoulder, through the tissues of the base of the neck on to the great vessels and atrium, the initial deflection remains negative or downward until the electrode actually touches the anterior right ventricle (6). It then becomes upright. Thus, although in the direct lead it is upward, it is a proximal effect and disappears with CO<sub>2</sub>. This paradoxical finding is not explained and is a subject of future research.

In standard limb leads, during administration of CO<sub>2</sub>, R is decreased and QS in-

creased because the factors tending to move the beam downward are increased and those which tend to move it upward are decreased. For example, let us consider lead II when CO<sub>2</sub> is in the right ventricle. Normally, in lead II the QRS deflection is upright when the left leg is positive and the right arm is negative. With CO<sub>2</sub> the right arm becomes positive and the left leg negative (see above). The initial ventricular deflection shows either a decreasing R and an increasing S or an actual QS under these experimental conditions.

Acute dilation of the right ventricle is produced by carbon dioxide. Equal dilation caused by rapid injection of blood or compressing the pulmonary artery does not produce similar changes in the electrocardiogram (1). Buchbinder and Katz found that acute distension causes no change in the electrical axis (8). The changes due to carbon dioxide occur with the first or second beat, before any change in blood pressure has taken place or before the left ventricle experiences any decrease in inflow.

### SUMMARY

When an animal is in a supine position the dielectric carbon dioxide placed in the right ventricle causes: *a*) reduced downstroke (QS) and increased upstroke (R) in VR; *b*) reduced downstroke (QS) and small increased upstroke (R) in VL; *c*) increased downstroke (QS) and decreased upstroke (R) in VF.

When the apex of the heart is shifted to the left, carbon dioxide in the right ventricle causes a large downward beam movement and a smaller upward one in VL. When the apex of the heart is shifted to the right, carbon dioxide in the right ventricle causes a larger upward beam movement and a smaller downward one in VL. These results are best explained by, and lend support to, a 'zonal interference' concept of the genesis of the electrocardiogram.

Extrasystoles induced by carbon dioxide in the right ventricle are downward in VR and upward in VF. In VR or VF, mechanically induced right sided epicardial extrasystoles and CO<sub>2</sub> induced endocardial extrasystoles have the same direction in any one lead under consideration.

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# DETERMINATION OF KINETIC ENERGY OF THE HEART IN MAN<sup>1</sup>

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IN CALCULATING the work of the heart, the kinetic energy factor is usually neglected. This omission is the result of the impression that the kinetic energy comprises but a small part of the total work of the heart. The kinetic energy of the heart in the laboratory animals studied has been variously estimated to constitute from 1 to 25 per cent (1) of the total energy of the heart. If these data can be applied to man, the kinetic energy becomes a factor of considerable importance and neglect of this factor would significantly underestimate the total work of the heart. Furthermore, even though the kinetic energy factor in man at rest may be small, this factor may increase significantly under conditions associated with a high cardiac output such as stress, exercise and anxiety, and in some types of intracardiac defects. Thus it has been established by cardiac catheterization in this laboratory (2) and elsewhere (3, 4) that the stroke volume of the right ventricle may remain high despite the presence of marked congenital pulmonary stenosis. The velocity of flow through the stenosed orifice must be greatly increased not only on this account but also because of the decrease in cross-section area. A similar phenomenon occurs in aortic stenosis.

Data and methods for the calculation of the total work of the heart in man are limited. This study was initiated in an attempt to establish the contribution of kinetic energy to the work of the heart in normal man utilizing techniques which have only recently become available, and which allow better definition of the individual factors concerned in the calculations. Only after the normal range in man is adequately defined, can the evaluation of the work of the heart under stress or pathologic condition be attempted.

Due to the limitations of available methods, it is obviously not feasible to obtain the total work of the heart in man by integration of the moment to moment pressure, output and velocity changes during the ejection period as was done in the turtle heart (5). Instead, approximation must be made by using the total output, and mean pressures and velocities in the calculations. It was previously pointed out (5) that the kinetic energy factor of the work of the heart is consequently underestimated more than the potential energy factor.

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<sup>3</sup> Sam Ehrlich Memorial Fellow

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<sup>5</sup> Fellow of the National Heart Institute

*Formulae.* It is agreed that the useful work of each ventricle is the external work (6). This consists of the pressure exerted upon the blood to be ejected and the velocity imparted to it; it includes the work done in circulating the blood through the coronary circulation as well as the closure of the semilunar valves (6). The total external work of the heart ( $W$ ) is obviously equal to the sum of the work of both ventricles ( $W_R$  and  $W_L$ ), viz.:

$$W = W_R + W_L \quad (1)$$

Simplified formulae have been adopted to define the work of each ventricle. Thus, the work of the right ventricle is

$$W_R = P_R V_R + \frac{M_R v_R^2}{2g} \quad (2)$$

where  $P_R$  is the mean pressure in the pulmonary artery just beyond the semilunar valves,  $V_R$  is the stroke volume of the right ventricle,  $M$  is the mass (stroke volume times the gravity of the blood (1.057 for men and 1.053 for women (7))),  $v_R$  is the average velocity of the blood at the root of the pulmonary artery during ejection, and  $g$  is the gravitational constant.

Similarly, the work of the left ventricle is

$$W_L = P_L V_L + \frac{M_L v_L^2}{2g} \quad (3)$$

where  $P_L$  is the mean pressure in the aorta immediately beyond the semilunar valves,  $V_L$  is the stroke volume of the left ventricle,  $v_L$  is the average velocity of the blood at the root of the aorta during ejection, and  $M$  and  $g$  are as above.

The total work of the heart thus becomes

$$W = \left( P_R V_R + \frac{M_R v_R^2}{2g} \right) + \left( P_L V_L + \frac{M_L v_L^2}{2g} \right) \quad (4)$$

It is assumed that  $V_R$  equals  $V_L$  and  $M_R$  equals  $M_L$  and so  $M$  and  $V$  are substituted for them. The velocity ( $v$ ) is directly proportional to the stroke output ( $V$ ) and inversely proportional to the cross-sectional area ( $A$ ) of the pulmonary artery or aorta and to the duration of ejection ( $T$ ). Consequently

$$v = \frac{V}{TA} \cdot k \quad (5)$$

by substituting the value of  $v$  given in formula 5 [and  $V$  for  $M$ ] in the formula for kinetic energy  $Ke = Mv^2/2g$ , the following formula is obtained

$$Ke = \frac{V^3}{A^2 T^2} K \quad (6)$$

In these two formulae (5) and (6),  $Ke$  is the kinetic energy and  $k$  and  $K$  are constants.

Certain facts become apparent from formula 6: the stroke volume ( $V$ ) is of paramount importance in determining the velocity factor because it is a large variable which is cubed in calculating the kinetic energy. The cross-sectional areas ( $A$ ) of the pulmonary artery and aorta are also significant factors not only because they are large and variable but also because their values are squared. On the other hand, the duration of ejection ( $T$ ) is a small and fairly constant value (about 0.25 sec.) and consequently of less significance.

#### METHODS AND MATERIAL

Only subjects known to have normal hemoglobin values and to be free of cardiovascular and pulmonary disease were chosen for this study. The patients were studied postprandially, at rest and without sedation. The conditions approached the resting state except for the excitement associated with the catheterization and angiocardiographic procedures.

Cardiac catheterization (8) and angiocardiology<sup>6</sup> (9) were used to obtain the data required by the above formulae. Mass was calculated from the stroke output ( $V$ ) as obtained by catheterization of the right heart. The duration of the ejection period ( $T$ ) was measured from the pressure curves recorded through a catheter placed in the main pulmonary artery directly above the pulmonic valve. A Hamilton manometer was used to record pulmonary arterial pressure. In some of the cases the Sanborn capacitance electromanometer was used. The potential energy of the left ventricle ( $P_L V$ ) was calculated with the assumption that the mean pressure in the root of the aorta exceeds that in the brachial artery by 35 mm. of mercury. The mean pressure in the pulmonary artery was measured directly from the pressure curve by dividing the sum of the systolic and diastolic pressures by two.

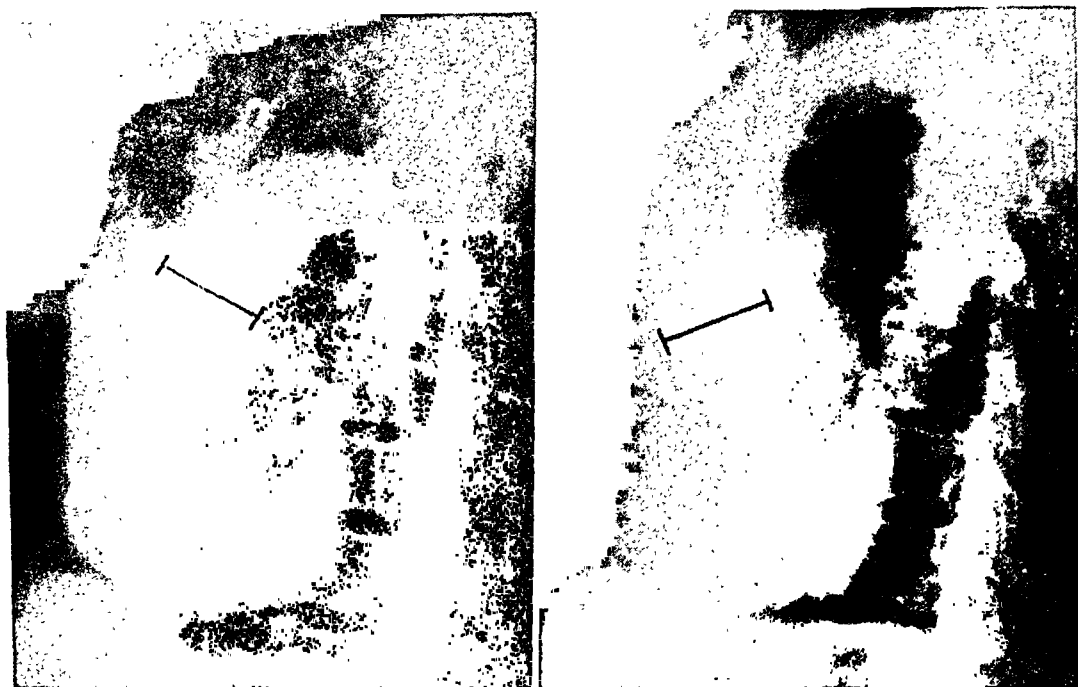


Fig. 1 (Left). ANGIOCARDIOGRAM of *patient 4* in left anterior oblique position showing (H) where the measurement of the diameter of the pulmonary artery is made. Discussed in text.

Fig. 2 (Right). ANGIOCARDIOGRAM of *patient 4* in left anterior oblique position showing (H) where the measurement of the aorta is made. Discussed in text.

Angiocardiology with the patient in the left anterior oblique position was used to measure the diameters of the pulmonary artery and aorta necessary for the calculation of the respective mean velocities. Measurements of these vessels as obtained at autopsy were deemed inadequate for the calculation of velocity of blood flow in the living state. In order to minimize the error arising from cyclic variations in diameter of these vessels, average values were obtained from serial angiograms for use in the formula. A fixed reference level for both vessels was used (figs. 1 and 2). The diameter of the aorta was measured approximately 2 cm. above the greatest diameter of the root of the aorta. The diameter of the main pulmonary artery was taken approximately 1 cm. below the bifurcation of its left and right branches.

To minimize the distortion in recording the measurements of the vessel diameters

<sup>6</sup> Diodrast used in these studies was obtained through the courtesy of Winthrop Stearns Co.

a perforated metal grid was constructed containing holes to mark off the corners of one-centimeter squares. A control roentgenogram of the chest was made. The grid was then placed at a distance from the cassette calculated to represent the plane of the patient's heart and the control film was re-exposed. The images of the grid holes were thus superimposed upon the control film. By determining the distortion of one marked centimeter, at the tube distance used, it became possible to correct for distortion of the vessel diameter under consideration in successive films.

### RESULTS

The angiocardigraphic measurements of the diameters of the pulmonary artery and aorta are indicated in table 1. Catheterization data and calculated kinetic energies for the right and left ventricles are summarized in table 2; all values are given per beat and in gram-centimeters.

The diameters of the pulmonary artery ranged from 2.3 to 3.5 cm.; those of the aorta from 2.3 to 3.1 cm. There is considerable variation in the size of both vessels, with no fixed ratio of aortic to pulmonary artery diameter. These data indicate the

TABLE 1. MEASUREMENTS OF DIAMETERS OF PULMONARY ARTERY AND AORTA IN SIX NORMAL SUBJECTS

PATIENT NO.	SEX	AGE	DIAMETER OF PULMONARY ARTERY	DIAMETER OF AORTA	CROSS SECTION AREA OF PULMONARY ARTERY	CROSS SECTION AREA OF AORTA
		yr.	cm.	cm.	cm. <sup>2</sup>	cm. <sup>2</sup>
1	M	36	3.23	3.10	8.23	7.54
2	F	37	2.70	2.30	5.71	4.14
3	F	15	2.32	2.75	4.23	5.98
4	M	56	3.50	4.35	9.60	14.92
5	F	26	2.50	2.90	4.90	6.59
6	F	34	2.62	2.42	5.60	4.60

large margin of error which may be introduced by application of mass postmortem measurements in the calculation of the work of the heart in an individual living subject. The possible errors in estimating vessel size in a given patient are further indicated by the large necropsy series of Suter (10), Kani (11) and Kaufmann (12) as well as the angiocardigraphic measurements of Dotter and Steinberg (13) and Vaquez (14). Age, body build and surface area seem to affect vessel size. Consequently, in view of the significance of the cross-sectional area in the calculations, dynamic measurements of the aorta and pulmonary artery in the individual under consideration must be employed in the calculation of the heart's work.

Two other factors to be considered in calculating the work of the heart are the duration of ejection and the diastolic pressure (15) in the vessel under consideration. The ejection period was found to be relatively constant and small, varying between 0.26 and 0.30 seconds. The diastolic pressure represents the magnitude of drainage from the peripheral branches of the main vessels, and is also relatively constant in the systemic circuit (ranging between 75 and 82 mm. Hg) and in the pulmonary circuit (ranging between 5 and 14 mm. Hg). Hence these two factors have little significance in modifying the value of the kinetic energy output.

As mentioned above the stroke output is the most significant factor in determining the kinetic energy of the ventricles. The values for stroke output in cases 3, 5 and 6 are closely grouped. The velocities vary between 33 and 46 for the pulmonary artery, and 29 and 40 cm/sec. for the aorta. The corresponding kinetic energy values for the right ventricle vary from 31 to 65, and for the left ventricle from 25 to 46 gm.cm. per beat. *Case 2*, with comparable vessel size, represents an example of a high stroke output (doubtlessly due to excitement). The comparison of this case with any of the previous group shows that the increase in stroke volume causes an increase in the kinetic energy of approximately 400 per cent for the right ventricle and 1200 per cent for the left ventricle. *Cases 1* and *4*, particularly the latter, represent a combination of high stroke output and large cross-sectional area of the vessels. Despite the relatively high stroke output, the velocity of flow and the kinetic energy for both ventricles were the lowest in the series.

TABLE 2. HEMODYNAMIC DATA OBTAINED ON SIX NORMAL SUBJECTS

PATIENT NO.	Stroke Output	Pulse Rate (beats/min.)	Mean Pulmonary Arterial Pressure	Estimated Mean Aortic Pressure	Duration of Ejection	RIGHT HEART				LEFT HEART				TOTAL OF BOTH HEARTS		
						Potential Energy <sup>1</sup>	Velocity in Pulmonary Artery	Kinetic Energy <sup>1</sup>	Kinetic Energy / Total Energy × 100	Potential Energy	Velocity in Aorta	Kinetic Energy <sup>1</sup>	Kinetic Energy / Total Energy × 100	Potential Energy	Kinetic Energy	Kinetic Energy / Total Energy × 100
	cc.		mm. Hg	mm. Hg	sec.	gm. cm. <sup>1</sup>	cm/ sec.	gm. cm. <sup>1</sup>		gm. cm. <sup>1</sup>	cm./ sec.	gm. cm. <sup>1</sup>		gm. cm. <sup>1</sup>	gm. cm. <sup>1</sup>	
1	104	52	14	135	0.26	1970	48.6	130	6.2%	19000	53.0	160	0.84%	21000	290	1.4%
2	94	78	11	141	0.26	1400	63.5	200	12.5%	17900	87.4	390	2.13%	19300	590	3.1%
3	58	80	13	133	0.30	1018	45.7	65	6.0%	10400	32.2	33	0.32%	11400	98	0.9%
4	89	70	18	123	0.28	2170	33.1	52	2.4%	14800	21.3	20	0.17%	16500	72	0.4%
5	57	74	12	129	0.30	923	38.8	46	4.7%	9900	28.8	25	0.25%	10900	71	0.6%
6	52	82	12	130	0.28	842	33.2	31	3.6%	9100	40.4	46	0.45%	10000	77	0.8%

<sup>1</sup> Per beat

As expected, the ratio, Kinetic Energy/Total Energy × 100, which is an expression of the proportion of energy utilized by the ventricle in imparting velocity to the blood, is much higher for the right than for the left ventricle, since the pressure in the pulmonary artery is only about one sixth that in the aorta.

#### DISCUSSION

It is obvious from this analysis that the kinetic energy is a significant factor in the work of the human heart. However, the accuracy of the methods used in this study for its determination is limited by the accuracy with which individual factors can be measured and by certain simplifications and basic assumptions. The catheterization technique is now well-established and the accuracy and reliability of the results when measured under properly stabilized conditions is generally accepted. The duration of ejection can also be obtained with reasonable approximation provided the basic principles of registration technique are not neglected (16, 17).

Errors may be introduced due to other factors involved in the calculation. The changes in diameter of the vessels during the cardiac cycle and the mechanical distortions of the angiocardigraphic measurements are only partly corrected with the procedures mentioned above in obtaining an average diameter at a chosen reference point. The elastic vessels respond to pressure changes during the cardiac cycle with a corresponding change in volume. In our group of cases the volume changes of the aorta were secondary to pressure changes ranging from 80 to 130 mm. Hg. This pressure-volume relationship has been thoroughly investigated by Hallock and Benson (18) in man using necropsy material. From the curves they plotted of the absolute volumes of aortas of different age groups against varying pressures, it can be seen that in the 30-year age group the volume increases per unit of length from 2.4 at a pressure of 75 mm. Hg to 2.9 cc. at 125 mm. Hg. In the 50-year age group the volume at 75 mm. Hg is much the same as in the 30-year group, increasing to 2.7 cc. at a pressure of 125 mm. Hg. They evaluated the application of these necropsy data to living patients of the same age using Moen's formula:

$$\% \text{ increase in volume per mm. Hg in pressure} = \left( \frac{3.57}{\text{velocity of pulse wave}} \right)^2$$

and found that the data in their *in vivo* series coincided with the data from necropsy aortas within 6.4 per cent. Further, when the 'elastic after-action' was considered, the average difference was only 2.6 per cent. No similar data are available for the pulmonary arteries. It may be assumed, however, that an error of the order of about  $\pm 10$  per cent may be introduced because of the phase of the heart cycle in which the vessel diameter is measured.

The assumption has been commonly made in calculating the kinetic energy that the flow is uniform in the cross-section of the aorta and pulmonary artery. This is, of course, not true theoretically, and recently Ralston and Taylor (19) have obtained data that confirm the view that there is a laminar flow through the dog's aorta, flow being greatest in the center and least in the part of the vessel adjacent to the vessel wall.

Differences in the values for stroke volume, size of vessels and velocity of flow between smaller animals and man raise the possibility that turbulent flow may exist in the root of the aorta and pulmonary artery in man. No experimental method has thus far been developed which can be applied to man to ascertain the basic pattern of flow in the aorta and pulmonary artery. An estimate of probability of turbulent flow may be reached by means of Reynolds formula. Reynolds has shown that if the velocity of flow through a given tube exceeds a certain critical level, turbulence may be expected. We have the formula  $Re = Dv/d$  (20), where  $D$  is the diameter of the vessel,  $v$  the mean velocity and  $d$  is the kinematic viscosity (0.0169) for blood. The value given by this calculation is in abstract terms and the critical number (limit) above which turbulence should be expected is given as 2000. In applying our data to Reynold's formula, we found that the values were far above this critical limit (table 3). This would imply the existence of a turbulent flow in the roots of both vessels.

If we accept this possibility, a clear cut differentiation should be made between the kinetic energy of the flowing blood, and the energy expended by the ventricles

in setting the blood into motion. The energy expenditure and the kinetic energy of the blood would no longer be equal, because a considerable part of the energy of the ventricles would be dissipated and lost as a result of the turbulence. This important distinction between the kinetic energy of the flowing blood and the energy expenditure of the ventricles was particularly stressed by Visscher (21). These factors of turbulence and parabolic flow are limiting factors in the accurate determination of kinetic energy. Both lead to an underestimation.

Finally, Katz (5) in the turtle heart has shown that the use of *mean* pressure and *mean* velocity in calculating the external work of the heart underestimates both forms of energy, and also alters the calculated distribution of the work between these two forms. He has also shown that the magnitude of error varies, and is influenced by various factors, such as augmentation of the initial volume, fatigue of the myocardium and type of contraction. These data, when translated to man, lead to the conclusion that the potential and particularly the kinetic energies calculated by us tend to be underestimated.

TABLE 3. REYNOLDS NUMBERS FOR PULMONARY AND AORTIC FLOWS

PATIENT NO.	PULMONARY FLOW	AORTIC FLOW
1	9000	10000
2	10000	12000
3	6000	6000
4	7000	6000
5	6000	5000
6	5000	6000

The use of the accepted formulae for the estimation of kinetic energy in congenital malformation of the heart (22) appears to us to be valid only in instances of isolated stenosis of the outlet valves. The presence of shunts and confluent flows in other congenital cardiac lesions create a situation for which the conventional formulae were not designated.

Our results of kinetic energy are significantly higher than those given recently by Bing (22). The discrepancy is due to the difference in formulae employed. The data given by Bing are: stroke output 67 cc.; area of pulmonary artery and aorta 5.14 cm.<sup>2</sup> and 3.9 cm.<sup>2</sup>, respectively. His data for the cross-sectional area is taken from necropsy material. The velocity of flow from the right heart, as calculated by Bing, was 43 cm/second, closely approximating our results. His velocity of flow from the left heart (57 cm/sec.) is greater than our calculated results due to his use of necropsy data. These yield a smaller cross-sectional area of the aorta than we have found in the living patient by angiocardiology. In calculating the kinetic energy, Bing used the formula:  $Mv/2 g$  whereas we used the generally accepted formula for kinetic energy  $Mv^2/2 g$ . As a result he gives 1.46 and 1.94 gm. cm/second as the kinetic energy of the right and left ventricles respectively. Applying Bing's data to the formula,  $Ke = Mv^2/2 g$ , one obtains 62 gm. cm/second for the right heart and 111 gm. cm/second for the left heart.

While we have made a serious attempt at calculating the kinetic energy and total work of the heart which newer techniques have permitted, it would only be proper to leave the impression that these are still approximations of the order of  $\pm 15$  per cent of the true values obtainable with mean values. As techniques improve these calculations should become more accurate. The results are sufficiently clear, however, to indicate that neglect of the kinetic energy is not justifiable in calculating the work of the heart in man particularly in the case of the right heart.

Recognition of the fact that stroke volume is by far the most important factor in determining the kinetic energy permits a rough index to be obtained of the kinetic energy of either or both ventricles. This can be calculated by cubing the cardiac output per minute and dividing by the square of the diameter of the aorta or pulmonary artery. Thus, with an output of 5 l/minute and a diameter of the aorta of 3 cm., the index would be  $125/9$ ; if the output were 10 l/minute with the same diameter, the index would be  $1000/9$ ; if the diameter were 2 cm. the index would be  $125/4$ .

#### SUMMARY

An approximation of the kinetic energy of the heart and velocity of flow in the aorta and pulmonary artery was made in 6 normal adults by means of angiocardiology and cardiac catheterization. The formulae used were  $Ke = Mv^2/2$  g. Velocity ( $v$ ) was calculated from the formula  $v = V/TA$  where  $V$  is stroke output,  $T$  is duration of ejection and  $A$  is cross-section area of the aorta or pulmonary artery.

The validity of the basic data and assumptions necessary for the calculations are discussed. The values for the kinetic energy expressed as a percentage of the total energy varies from 2.4 per cent to 12.5 per cent for the right heart, and from 0.25 per cent to 2 per cent for the left heart. Stroke volume is by far the most important factor in determining the kinetic energy but other factors such as the caliber of the aorta and pulmonary artery are important.

The intrinsic limitations of the assumptions and calculations suggest that our data may underestimate the energy expended by the ventricle in imparting velocity to the blood. The kinetic energy increases markedly under stress. Neglect of this factor will therefore significantly underestimate the work of the heart of man.

The existence of turbulent flow in the roots of the aorta and pulmonary artery are suggested by the high Reynolds numbers obtained. As a direct corollary, distinction is made between the kinetic energy of the blood in the roots of the great vessels and the energy expended by the respective ventricles in setting the blood into motion; the latter being the greater.

An index of kinetic energy is suggested which gives a rough idea of the order of change with variation in cardiac output and with changes in blood vessel caliber.

We are indebted to other members of the Department for assistance in carrying out the observations.

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# REPOLARIZATION IN THE DOG VENTRICLE: EFFECTS OF HEATING AND COOLING ENTIRE EPICARDIAL SURFACE<sup>1</sup>

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THE contribution of the subendocardial myocardium to the electrocardiogram has recently become a subject of renewed interest. An answer to this problem has been sought in a number of ways both experimentally and clinically. Lewis (1) found by eliciting premature systoles from points on the endocardium and epicardium of the right ventricle which faced each other, that the epicardial premature systole consisted solely of an R wave and the endocardial premature systole had a Q preceding the R wave. This demonstration led to the conclusion that an endocardial-epicardial potential difference developed during depolarization which was reflected in the electrocardiogram. By similar reasoning the T deflection of the electrocardiogram was thought to be produced by differences in onset and rate of return of the endocardial and epicardial ventricular muscle from the excited to the resting state.

This concept was applied to an analysis of the limb leads by Gardberg and Ashman (2), amplified by Bayley (3), and extended to the chest leads by Wilson and his associates (4-7). The latter regarded the V precordial leads as semi-direct leads from the anterior ventricular surface (7). Although generally in accord with the deductions of Wilson, Katz and his associates (8-10) have taken exception to this meaning of the chest leads. Studies of the distribution of the surface potential over the chest during various phases of registration of QRS demonstrated that the surface fields were asymmetric and showed peculiar concentrations and dispersal of potential lines. They were unable to substantiate any major preferential depiction by the precordial leads of events in regions of the heart beneath the chest electrode, and concluded that the chest leads record at any given point "the orderly alteration of the electrical field created by the passage of the impulse throughout the heart (and the restitution process) without the exertion of undue influence by the activity of the region of the heart beneath the electrode upon the potentials the latter records" (11).

Nahum and Hoff and their associates (12-16), in a series of experiments have more precisely defined the field theory in terms of specific regions of the heart. By means of impressed potentials they were able to delineate the the surface of the heart into two zones, a proximal and distal, which when made negative with respect to the rest of the heart resulted in a downward or upward deflection in the lead of the reference electrode, limb or precordial. They found that premature systoles experimentally induced in any part of the proximal zone, endocardial or epicardial, were identical in contour. The same was true of the distal zone, but the premature systoles were in a direction op-

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posite to those in the proximal zone. An earlier demonstration of the existence of such zones based upon the contours of epicardial premature systoles is to be found in the papers of Abramson and Weinstein (17) and Abramson *et al.* (18) whose results correspond precisely with those reported later by Nahum, Hoff and their associates. Nahum, Chernoff and Kaufman (13-15) also determined that S-T elevations occurred only with surface injury in areas constituting the proximal zone and S-T depressions with injury in the distal zone. And finally (13-15), by local cooling and heating, they found that the T wave became more positive when repolarization was accelerated in the proximal zone or slowed in the distal zone; conversely, the T wave became more negative when repolarization was slowed in the proximal zone or accelerated in the distal zone. It is of interest that in an earlier study by Hoff and Nahum (19) altering repolarization by the same thermal methods yielded qualitatively opposite effects on the T wave in the left as compared with the right ventricle, but identical results everywhere on the surface of the same ventricle without regard to proximal or distal zones. From all these experiments Nahum and Hoff concluded that an endocardial-epicardial potential difference during activity contributes nothing to the electrocardiogram, and that each limb or precordial electrocardiogram represents the algebraic summation of forces developed during excitation and recovery of proximal and distal zones of the heart relative to the reference electrodes.

This apparent controversy led to our reinvestigation of this problem. In considering an approach to the study of the role of the subendocardial myocardium to the genesis of the electrocardiogram, the method of perfusing the pericardial sac seemed to be a ready means of changing the temperature of the entire epicardium relative to the endocardium, quantitatively and temporally to approximately the same extent, with a minimum of trauma to the myocardium and Purkinje system. It has the advantage of avoiding the inherent pitfalls which are present in drawing inferences as to the behavior of the whole heart from experiments in which but local changes are induced. We share with Byer, Toth and Ashman (20) the opinion that principles which operate during repolarization may apply also to changes occurring during depolarization.

#### METHODS

Thirty-five experiments were performed on 7 dogs. The procedure consisted of heating and cooling the entire epicardial surface and recording the effects upon the electrocardiogram in the standard and aV limb leads. Each animal was deeply anesthetized with sodium pentobarbital (25-35 mg/kg. of body weight). The chest was opened through the 5th intercostal space, and respirations maintained artificially.

Cooling and warming of the entire epicardial surface was accomplished by perfusing the pericardial sac with isotonic saline solution of varying temperatures (42°, 50°, 30°, 20°, 15°C.). The solution was introduced and drained via two flanged glass cannulae inserted into the pericardium to provide a water-tight inlet and outlet. A small rubber sleeve was pushed down over each cannula to seal the pericardium to the flanged end.

Following a control electrocardiogram, saline solution at 37°C. was allowed to run into the inlet cannula from an overhead container at a rate of 75 cc/minute until 200 to 250 cc. had been circulated through the pericardial space. During this procedure a second electrocardiogram was taken to compare with the first in order to note possible effects produced by perfusion with normal saline at body temperature. Following this control experiment, the pericardial sac was perfused with saline solution of varying temperatures, during which time the electrocardiograms were taken. Each subsequent experiment was performed only after restitution of the electrocardiogram

had occurred. Extremes of temperature were arbitrarily limited to the point of producing S-T segment changes. A direct writing electrocardiograph<sup>4</sup> was used. In every instance both standard and aV limb lead recordings were made before, during and at varying intervals after epicardial temperature alteration.

Pericardial tamponade was avoided by providing a large outlet which permitted the free escape of fluid from the pericardial sac. The rate of perfusion was maintained relatively constant throughout all the experiments on any one animal. Complete and rapid circulation of the perfusion fluid was effected by the churning action of the heart itself. The almost immediate appearance and quantitative recovery of the fluid at the outlet was evidence of the efficiency of this pumping action. The temperature of the fluid while circulating through the pericardial sac was little different from that at the time of entrance, since it changed less than 2°C. when recorded at the outlet.

### RESULTS

*Warming Entire Epicardial Surface.* Typical changes are shown in figure 1. The standard limb leads showed like changes with all degrees of heating in 5 of the 7 animals studied. In these experiments, warming the epicardial surface caused the T waves to become more positive, the degree of change from the control tracing varying directly with the temperature of the saline perfusate. In the remaining 2 animals, warming caused the T wave to become more positive in leads II and III, but negative in lead I. In one of these, the change in lead I was slight, in the other an upright T became definitely inverted. Warming the epicardium caused consistent changes in the aV leads in all animals studied; the T wave became more negative in aVR and aVL, and more positive in aVF. As in the case of the standard limb leads, the degree of change in direction of the T wave was directly proportional to the temperature of the saline solution used.

In 2 animals quantitatively significant S-T deflections occurred with maximal epicardial warming. In these instances the direction of S-T deviation in all leads was always opposite to the induced direction of the T wave. In no instance did the degrees of warming used cause the duration of QRS to change by more than 0.01 seconds. Warming of the heart surface occasionally produced slight changes in the amplitudes of QRS when saline solution of 50°C. was used. The direction of QRS and its phases remained constant throughout the warming experiments in each animal, even though they were slightly altered in amplitude by maximal warming. Thus the changes in the net area of QRS were negligible with warming of the epicardium. The heart rate changes that occurred with epicardial heating were also minimal. Warming experiments had essentially no effects on the duration of the Q-T interval.

*Cooling Entire Epicardial Surface.* Typical changes are shown in figure 1. Cooling of the epicardial surface caused the T waves in the standard limb leads to become more negative in 5 of the 7 animals studied. The degree of induced negativity was directly proportional to the coldness of the saline solution used. In the other 2 animals cooling caused the T wave in leads II and III to become more negative and the T in lead I to become more positive. These 2 dogs were the same in which the changes in T in lead I were opposite to that in leads II and III when the epicardial surface was warmed. In 5 animals, cooling caused the T wave to become more positive in aVR and

<sup>4</sup> Sanborn Viso-Cardiette.

aVL, and more negative in aVF. The T wave became more negative in aVL in the remaining 2 animals. These 2 dogs were not the same as those in which discrepant changes were found in lead I.

Extremes of cooling consistently induced S-T deviations in the opposite directions to the T wave change. In 2 animals cooling the epicardium with a saline perfusate of 15°C. caused the duration of QRS to increase a maximum of 0.015 seconds. The QRS duration did not change more than 0.01 seconds in any of the other experiments of epicardial cooling. Maximal cooling caused a slight alteration in the amplitude of QRS; cooling with saline solution at 30°C. caused minimal and negligible

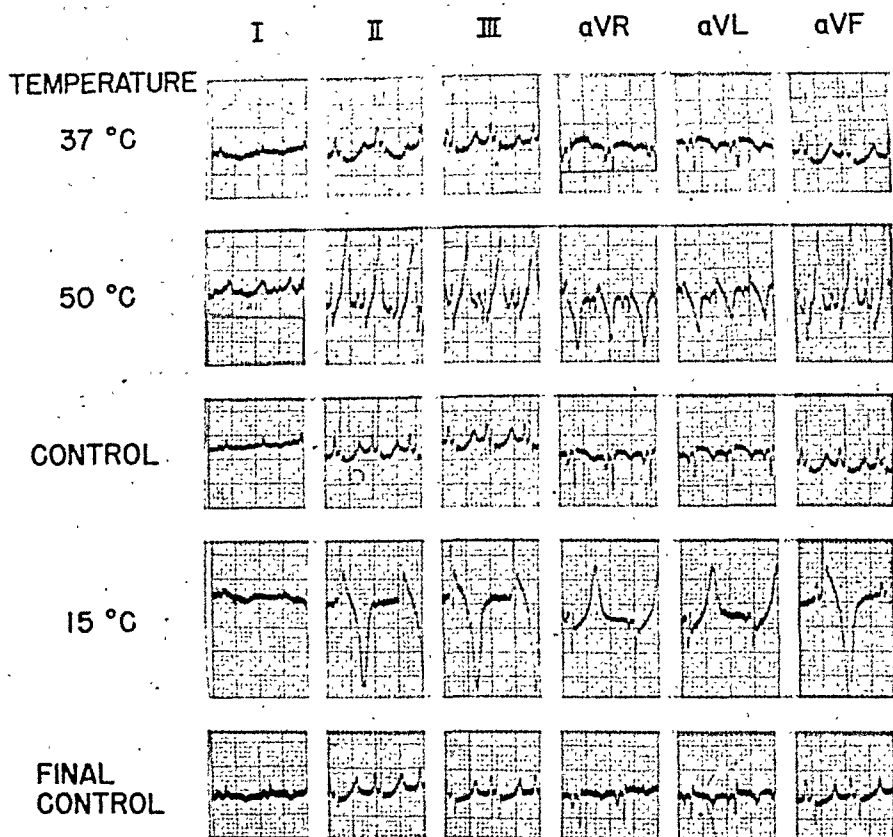


Fig. 1. TYPICAL EXAMPLE of one experiment illustrating results of only extremes of temperature employed. Heating caused the T wave to become more positive in the standard limb leads, more negative in aVR and aVL, and more positive in aVF. Cooling caused the T wave to become more negative in the standard limb leads, more positive in aVR and aVL and more negative in aVF.

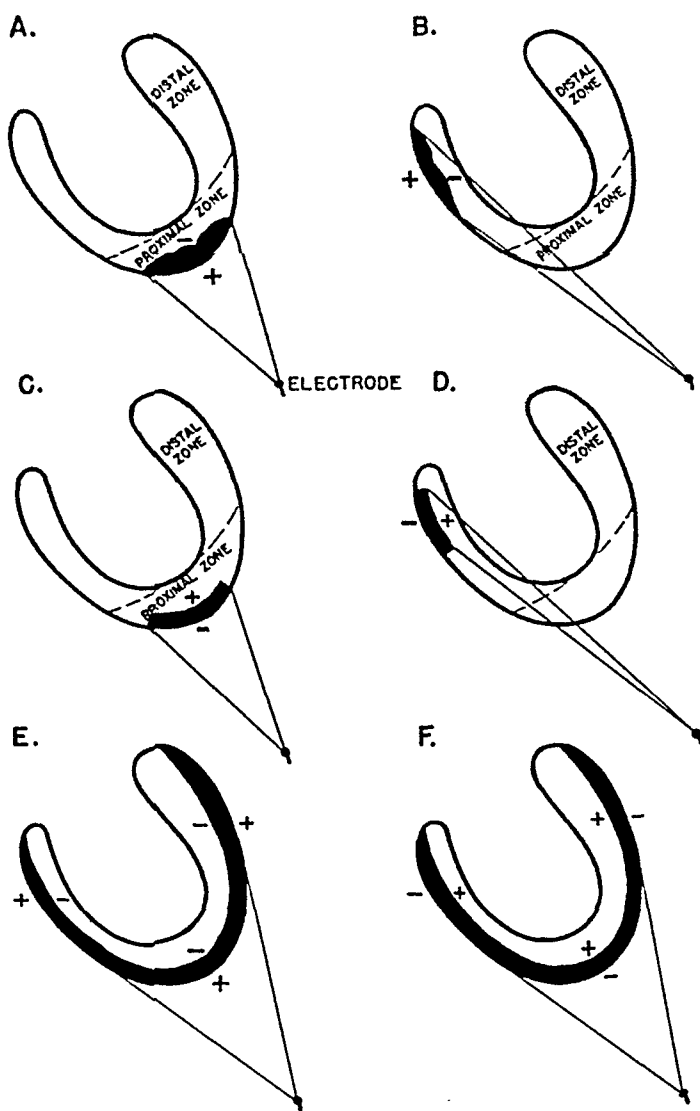
changes. As with warming, the phases of QRS were not altered significantly either in contour or temporal relationship. Moderate epicardial cooling caused slight slowing of the heart rate. Perfusion with solutions at 15°C. caused definite cardiac slowing. The duration of the Q-T interval was increased in direct proportion to the coldness of the perfusate, with the greatest increment occurring when the temperature of the saline solution was lowered to 15°C. from 20°C.

#### DISCUSSION

A relatively simple concept can adequately explain the changes observed in the T waves in these experiments. It is generally accepted that a cooled surface of the

heart is electrically negative with respect to an uncooled surface during activity. Our results during inscription of the T wave coincided with predictable changes which theoretical considerations based upon the above principle would lead us to anticipate; namely, cooling of the entire epicardium led to a reversal in direction of the T wave where it was previously upright and to diminished negativity or reversal of direction where it was originally inverted. Conversely, heating the epicardium led to an in-

Fig. 2. DIAGRAMMATIC REPRESENTATION of effects of induced local and general changes in subepicardium on repolarization. In graphs A, B, C and D the dash line separates proximal zone (below) from distal zone (above). In all graphs the septum is omitted for simplicity and only the thinner right and thicker left ventricle is shown (in a large electrically conducting medium) together with an exploring electrode in the outside medium (connected in such a way as to be approximately unipolar). Graphs A and B show that the direction of displacement of S-T in the proximal and distal zones as reported by Nahum and Hoff is a consequence of the particular injury interface which is oriented toward the reference electrode. Graphs C and D show how this same principle applies to directional changes in the T wave with cooling of the epicardium in the proximal and distal zones. E shows how heating the entire epicardium causes the T wave to become more upright. F shows how cooling the entire epicardium causes changes in T wave opposite to that in E. This entire handling is based on the same principle employed in a previous report from this department (22).



crease in height of the T wave where it was previously upright and to diminished negativity or reversal of direction where it was originally inverted. It is apparent, under the condition of these experiments in which temperature changes everywhere in the subepicardium were essentially the same, that only an endocardial-epicardial gradient of potential difference developing during repolarization could account for the observed alterations in the direction of the T wave. It is of little moment so far as the net effect is concerned whether one cools the epicardium or heats the endo-

cardium, or the converse. Therefore, our results are as readily interpreted in terms of an altered electrical state of the endocardium as of the epicardium.

Objections to a study of this nature because of the possibility of introducing artifacts such as air pockets, displacement of the heart, or T-wave changes incident to cooling of the heart surface by open-chest technique are irrelevant to our results: repeated control records were taken before and after each increment of temperature change, and only after restitution to previous control contours had occurred were subsequent alterations produced.

It is of interest that cooling the epicardium regularly prolonged the Q-T interval, but, contrary to the experience of Byer *et al.* (20) and Nahum and Hoff (21) changes in the net area of QRS were, on the whole, negligible except with extremes of cold. The explanation for this is not apparent in view of the fact that the differences in net area of QRST were such as to indicate the effect of cooling was considerable.

Pertinent to the problem of the significance of the subendocardial myocardium is the recent study by Hellerstein and Katz (22) concerning the effects of subepicardial and subendocardial injury. Employing direct epicardial and intracardiac electrodes they demonstrated that during the activated state the junction of the injured and uninjured regions was negative; the junction of injured area with the heart surface (endocardium or epicardium) was positive. An electrode directed toward the former interface registered S-T depression; one fronting on the latter interface registered S-T elevation. By reference to figure 2 it can readily be seen that the results of Nahum, Hoff *et al.* with respect to epicardial injury in proximal and distal zones can be entirely explained by the spatial orientation of the exploring electrode with respect to the region of the injury. It will be noted that any subepicardial injury in the proximal zone will be oriented to the reference electrode in a manner to produce upward displacement of S-T; subepicardial injury anywhere in the distal zone will be oriented to the reference electrode to produce downward displacement of S-T. A similar analysis is applicable to the T-wave changes with cooling and heating the epicardium produced both in the manner of Nahum and Hoff (13-15) and by our method, or by the method of Byer *et al.* (20) for the endocardium. It may therefore be stated that the findings of Nahum and Hoff show no exception from classical concepts, nor does their work necessarily deny an endocardial-epicardial gradient.

Finally, the clinical studies with intracardiac electrodes of Hecht (23) and of Battro and Bidoggio (24), and the case reports of subendocardial infraction by Langendorf and Kovitz (25), Price and Jones (26), Bayley (27) and Pirani and Schlichter (28), further point to the fact that subendocardial myocardium is not a silent zone.

It can be concluded that when the endocardial change is great, its altered electrical state conspicuously modifies the electrocardiogram.

#### SUMMARY

Cooling and heating the entire epicardium of the dog ventricles produced opposite effects on the direction of the T wave. Heating the epicardial surface caused the T wave to become more positive, whereas cooling caused the T wave to become

more negative in the standard limb leads. Heating caused the T wave to become more negative in aVR and aVL, more positive in aVF; cooling caused an opposite effect in these leads. These experiments demonstrate the existence of an endocardial-epicardial gradient during repolarization.

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# ACTION POTENTIAL OF HEART MUSCLE<sup>1</sup>

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THE action potential of heart muscle has been the subject of study for many years and is measured routinely as the electrocardiogram. As a result of the heterogeneous arrangement of muscle fibers within the muscle it is difficult to judge the true form of the action potential from measurements of the potentials generated by the heart during contraction or even of the potentials measured from heart muscle strips. However, on the basis of a great deal of indirect evidence and a little direct evidence, it has been generally concluded that heart muscle, unlike skeletal muscle, remains depolarized for a relatively long time following excitation and then rapidly re-polarizes.

If one accepts this interpretation, there are still two possible explanations of such behavior. The first is that each individual heart muscle fiber remains depolarized for the period of contraction. The second is that the individual fibers behave very much like skeletal muscle fibers, but in the muscle mass undergo excitation serially to give an average muscle depolarization lasting a relatively long time. There is virtually no evidence for or against either of these possibilities but from a study of smooth muscle and heart muscle strips Bozler (1) concluded that the latter mechanism was quite likely.

The present work was undertaken in an attempt first to furnish direct proof of the long-continued depolarization of heart muscle during contraction and, second, to discover whether or not this is a true cellular process.

## EXPERIMENTAL METHOD

The turtle ventricle was used exclusively in this study. It has been shown that the turtle ventricle has no coronary circulation and that the muscle fibers contract in the blood contained within the ventricle. Thus the ventricle is much like a sponge, and when the muscle contracts the blood is squeezed out. Garrey (2) pointed out that there are often quite long strands of muscle fibers within the ventricle which seemed to be relatively free. Upon investigation it was found possible by careful dissection under a dissecting microscope to free these fiber bundles from the rest of the muscle mass and by careful teasing reduce the bundle to about a hundred fibers. This made a strand of fibers about 0.5 mm. in diameter and perhaps 10 mm. long. Some of these bundles were fixed and stained. They showed that the muscle fibers were long and parallel and the majority of them reached the entire length of the strip. There were remarkably few anastomoses between fibers.

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Thus this preparation afforded an opportunity to study the muscle under conditions entirely comparable to those under which skeletal muscle has been studied, and it should be possible here to say with some certainty that the effects observed with this preparation are the same as those which would be observed if it were possible to isolate a single muscle fiber.

The arrangement for stimulation and electrical and mechanical recording is shown in figure 1. A relatively large piece of heart muscle is suspended between the two hooks in the lucite dish under saline, and the muscle carefully dissected until a suitable bundle is found. This is then carefully freed from the rest of the muscle and each end placed on the hooks. One hook is fixed to the dish and the other is supported by a transducer tube to measure muscle tension.

There are copper cooling coils, not shown in the figure, through which water at about  $9^{\circ}\text{C}$ . is circulated. This keeps the temperature of Ringer's about  $10^{\circ}\text{C}$ . At this temperature there is very little spontaneous activity and the muscle will stay in very good condition for many hours. One does not normally see spontaneous ac-

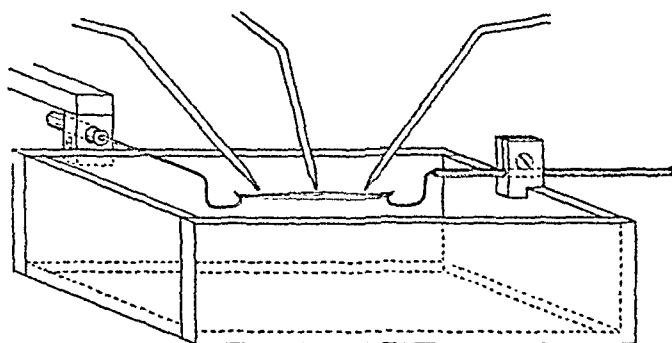


Fig. 1. ARRANGEMENT for stimulation and electrical and mechanical recording from heart muscle bundles.

tivity in the turtle ventricle, but these small strips, being somewhat stretched, will contract spontaneously for hours if the temperature is elevated to about  $12^{\circ}\text{C}$ . or higher.

An electro-mechanical transducer tube (R.C.A. type 5734) was used for mechanical recording as previously described (3). In this tube the plate can be moved through a flexible diaphragm, causing a change in tube characteristics and a consequent change in plate current, and the changed plate current will persist as long as the force is applied. The change is amplified by a D.C. amplifier and applied to a mirror oscillograph for recording on photographic paper. The transducer tube was mounted on a micromanipulator so the tension on the muscle could be regulated.

Stimulation and electrical recording was done through the micropipettes shown in figure 1. These have an inside diameter of about 0.2 mm. at the tip and are fire-polished. They were carried each on a separate micromanipulator so they could be held at any point on the muscle. The micromanipulator carried a holder which contained a well filled with Ringer's fluid into which the micropipettes protruded and also into which silver-silver chloride electrodes dipped.

Stimulation was by means of an electronic stimulator which delivered single

square wave shocks to the tissue through an isolation transformer. A micropipette at one end was made the cathode and the hook adjacent to it made the anode.

Electrical potentials were recorded from the remaining two micropipettes. They were merely placed very close to the muscle, but not touching it. They were connected, through the silver-silver chloride electrodes, to a differential D.C. amplifier, which was connected to a mirror oscillograph for recording on photographic paper. The photographic record then contained both the simultaneous electrical and mechanical events as well as a time marker. The records were taken in such a way that there was as little movement as possible of the muscle relative to the recording electrodes during contraction, and in every instance the muscle was moved relative to the electrodes an amount comparable to the movement during contraction, to prove that the electrical records contained no mechanical artifacts.

Electrical records taken with the muscle in saline were very small and the interpretation difficult. During the latter part of the work almost all records were taken with the muscle contracting in mineral oil. The muscle bundle was prepared as described above and then mineral oil was poured on top of the saline and the saline gradually drained off. This left the muscle in oil with a thin coating of saline around it which was continuous with the saline in the micropipettes. Muscle bundles would remain active in oil for many hours.

The injury potential was measured by crushing the muscle near one end with fine forceps and placing one microelectrode on this crushed region and the other on an active region. This was found to be much better than the technique of putting a noxious agent on one end of the muscle bundle because distances were so short that diffusion to the active electrode took place almost immediately and affected the results. However, the crushing technique was far from perfect, because it was difficult to tell when the tissue was completely crushed. Also, the crushed end would rather rapidly heal over, so the measured injury potential would become zero a very few minutes after crushing, in the face of excellent evidence that the muscle was in good condition under the active electrode. It was thus necessary to make the measurements very quickly after the crushing, but even so it is doubtful if any measurements were made on a fully 'killed end.'

The technique of measuring the injury potential was to crush the muscle and quickly place both electrodes on the injured region to establish the zero of potential and a short record taken. One electrode is then moved to an active region and another short record taken. The muscle is then stimulated and the 'monophasic' action potential is recorded. The electrode is again moved from the active region to the crushed end to re-establish the zero of potential. If there had been an appreciable drift of the zero, the measurement was discarded.

#### RESULTS

A record of the injury and the 'monophasic' action potential is shown in figure 2. Here the injury potential is 3.0 millivolts. When the action potential reaches the active electrode the injury potential falls approximately to zero and stays close to zero for more than 3 seconds and then rather rapidly returns to its former value.

Since there was so much fluid shunting the muscle fibers, these potentials probably represent only a small fraction of the true membrane resting and action potentials. In this figure the potential reverses by a fraction of a millivolt. No significance can be attached to this, since the measuring error could easily be this large.

In these records the response is usually all-or-none, indicating that there are enough anastomoses between fibers to make even this preparation syncytial. However, in a few instances a response was found which was not all-or-none, indicating

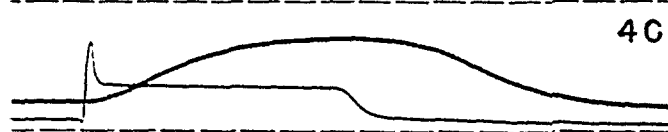
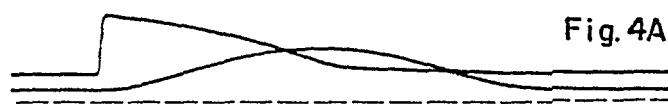
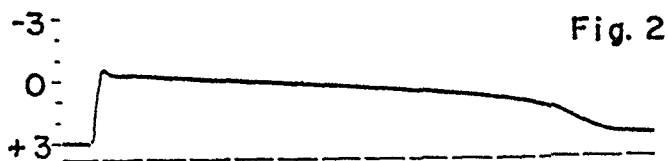


Fig. 2. ACTION POTENTIAL of heart muscle recorded between an electrode on active muscle and one on a crushed end. Scale at the left shows the potential in mv. of the active electrode relative to the one crushed end. Time marks 3/sec.

Fig. 3. ACTION POTENTIAL from a very small bundle of muscle cells, recorded with one electrode on active muscle and the other on a crushed end. Time marks 3/sec.

Fig. 4. THREE TYPICAL ACTION POTENTIALS (rapid response) and simultaneous contractions (slow response) from different heart muscle bundles. One electrode on active muscle and the other on a crushed end. Time marks 3/sec.

Fig. 5. SIMULTANEOUS ELECTRICAL AND MECHANICAL RECORDS from heart muscle bundles. A, both electrodes on active muscle. B, one electrode on active muscle and one on the muscle distal to a crushed region taken about 30 minutes after crushing. The crushed region was completely inactive, so the muscle fibers must have healed over proximal to the crushed region. Time marks 3/sec.

that sometimes a small bundle would become functionally isolated from the rest of the fibers by the process of dissection.

Thus it seems quite certain that the individual muscle cells become depolarized on excitation and remain so for a relatively long time, and then rather rapidly become re-polarized. In one experiment the muscle bundle was carefully teased away until there were only about 12 muscle fibers left. The 'monophasic' action potential is shown for this muscle in figure 3 and there is no indication that the depolarization is sustained by asynchronous action of the different muscle cells.

In figure 4 are shown three records demonstrating the relation between the

mechanical and electrical events. It will be observed that the tension continues to develop as long as depolarization persists, and does not start to subside until repolarization has taken place. These records also show the effect of partial healing of the injured end, especially in the second and third records. It is very difficult to get records like the one in figure 2, but there is every reason to believe that if all the records were taken with a perfectly killed end, they would be similar to figure 2.

When both electrodes are on active muscle a record similar to the top record of figure 5 is obtained. This gives a perfectly monophasic record usually followed by a T wave. This record is in complete accord with the record of figure 2. In these records the T wave is sometimes positive, sometimes negative and sometimes absent. Since the T wave is formed by the difference in rate of re-polarization of the muscle under the two electrodes, it follows that re-polarization may take place first at either end of the muscle strip, or the entire muscle strip may re-polarize at exactly the same rate. This seems clearly to indicate that the T wave is not a conducted response.

The second record of figure 5 was taken with one electrode on active muscle and the other beyond a crushed region which had 'healed.' The impulse did not pass the injured region but since there was no injury potential the record was essentially the same as if both electrodes had been on active muscle. This is also in complete accord with the other records.

#### DISCUSSION

There has been considerable evidence that the depolarization of heart muscle is prolonged, and lasts for a large fraction of the contraction phase, and that the T wave is caused by rapid repolarization (4-7). The present work is in complete accord with this concept and gives direct evidence that this is true at the cellular level.

The concept suggested by Bozler (1) that "the slow potential change is equivalent to a burst of impulses fused into a continuous state of negativity" is an intriguing idea but seems to have no basis in fact as far as the turtle heart is concerned. The action potential of heart muscle seems to be different from that of skeletal muscle in that re-polarization does not follow depolarization immediately but there is a time delay mechanism which delays re-polarization for a relatively long time. There is no reason to believe that the mechanism of conduction of the impulse along the fiber is any different from that in skeletal muscle and nerve.

#### SUMMARY

By microdissection methods it has been found possible to isolate small, homogeneous bundles of muscle fibers from the turtle ventricle in which the fibers are all parallel and there are very few anastomoses between fibers. Recording in mineral oil between a crushed end and an electrode on active muscle gave a resting potential as high as 10 millivolts. The conducted response causes the potential under the active electrode to fall to zero and remain there for as long as 3 seconds before suddenly re-polarizing. Muscle tension continues to develop throughout the period of depolarization, and relaxation does not start until re-polarization is practically com-

plete. It is concluded that the long-continued depolarization is a true cellular phenomenon.

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# EFFECTS OF INHIBITOR COMBINATIONS ON MEMBRANE POTENTIALS OF THE SYNOVIALIS

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THE study of the effects of metabolic inhibitors on potentials of the synovial membrane has led to an interpretation of the results on the basis of oxidation-reduction processes occurring in the cytochrome system, the principal electron conducting mechanism involved in cellular respiration (1-3). A large number of primary inhibitors have been studied, as well as a number of combinations of inhibitors, especially combinations of cupric ions and thiols, applied successively to the tissues. The results have shown that heavy metal and thiol inhibitors behaved antagonistically, each tending to abolish the positive potential produced by the other. By interpreting the positive potential as an increase in the mean potential at which electrons are accepted by the cytochromes, the correlation between the primary potentials of various thiols and their effect on the cupric potential could be explained electrochemically (3). By the method of combination of inhibitors, it is thus possible to determine in more complete detail the mechanisms of inhibition.

## EXPERIMENTAL RESULTS

The method of determination of synovial membrane potentials in the dog has been described in previous reports (1-4). For the study of combined inhibitors, the technique used has been the successive application of each inhibitor in isotonic NaCl (0.15 M) for a standard time interval during which several potential readings were taken. Usually duplicate solutions were applied for a total of three minutes during which time the potential was determined every thirty seconds (3). That procedure has been followed in obtaining the results to be reported.

The results are represented in figures 1 and 2, in which the ordinates represent the membrane potential in millivolts, taken as the average over the period in which duplicate solutions were applied with readings taken every thirty seconds. The abscissae denote time in minutes taken from the beginning of the experiment. Previously the potential had been stabilized by several rinsings with isotonic NaCl, during which it had been maintained constant within 1 or 2 millivolts.

*Combinations of Ferric or Cupric Ions with Cyanide or Pyrophosphate.* The results are shown in figure 1. In figure 1A it is shown that the potential given by 0.015M NaCN is increased by the application of  $7.5 \times 10^{-4}$  M ferric chloride. In two cases illustrated, figure 1B showed a very high cyanide potential (400 mv.) resulting from a combination of cupric and cyanide inhibition, while the other, figure 1C showed an antagonism between the two inhibitors with a decrease in the observed

potential. With pyrophosphate application the ferric ion potential was considerably lowered (fig. 1D), but the cupric ion potential was not significantly affected (fig. 1E).

*Combination of Iodide Ion with Various Agents.* The highest potentials found on any system to date have been those resulting from the successive application of isotonic sodium iodide and  $7.5 \times 10^{-4}$  M  $\text{CuCl}_2$  in isotonic NaCl. Figure 2A shows a typical result on this system. Following an initial potential of 25 mv. with NaI, which is its normal value, two subsequent rinsings, after alternating with  $\text{CuCl}_2$ , showed potentials of about 375 mv. and 500 mv. Subsequent rinsing with isotonic NaCl lowered the potential to about 375 mv.

The effects of mixtures of cupric and iodide ions are shown in figure 3 in comparison with the individual ions. Up to concentrations of  $10^{-6}$  M the effects are small, but at  $10^{-4}$  M and higher concentrations the added iodide greatly enhances the effect. At these concentrations the effect is far more than additive. It has been found previously that  $10^{-4}$  M is a concentration at which high potentials begin to be developed by heavy metal oxidants at the synovialis.

A rather similar effect has been observed with ferric ion in combina-

tion with iodide. In figure 2B it is shown that the addition of  $7.5 \times 10^{-3}$  M NaI to the ferric chloride solution increased the potential by about 150 mv. Synovial potentials in this system, however, have not been observed to approach the value 500 mv. observed in the copper-iodide systems.

With mercuric chloride as inhibitor, a rather different result is obtained by alternating with isotonic NaCl and NaI, the effect of which is shown in figure 2C. The initial iodide potential was the usual 25 mv., while the initial  $\text{HgCl}_2$  potential, following two washings with NaCl, was approximately 100. This potential was slightly increased by washing with NaCl. The next iodide potential started at 200 but fell rapidly to 50 mv. Repetitions of the sequence produced very similar effects, with a characteristic 'spike' indicating the unstable high initial potential given by iodide. The 'spike' appeared only on the first iodide washing after application of  $\text{HgCl}_2$ .

Figure 2 (D, E, F) shows respectively the effects of p-chloromercurio-benzoate, glutathione and isotonic sodium thiocyanate when alternated with isotonic NaI. The first substance is an inhibitor of sulfhydryl groups (5), the second is a sulfhydryl activator, while thiocyanate is a pseudo-halogen, a complex ion with outer electron

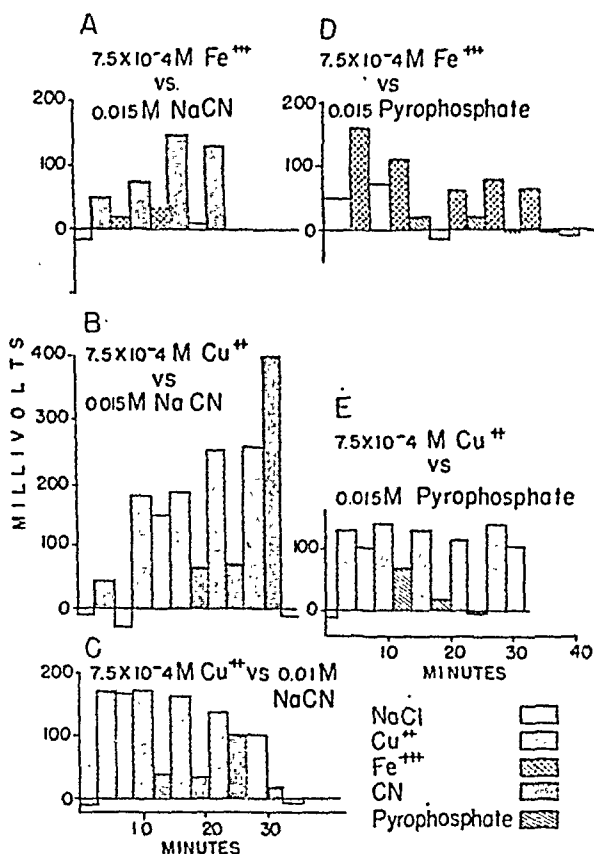


Fig. 1. EFFECTS of inhibitor combinations on membrane potentials. Heavy metal series.

shell comparable to that of a halogen. The iodide potential was found to be reversed by the sulfhydryl inhibitor and to be augmented by the previous application of glutathione. The latter effect was often very striking, the iodide potential increasing by 80 or 100 mv. when iodide was applied after a 3-minute treatment with 0.01 M glutathione. As shown in figure 2E the effect was maintained for several independent iodide rinsings alternated with NaCl for as long as 45 minutes after the application of glutathione. Figure 2F illustrates the characteristic behavior of thiocyanate when alternated with iodide. Quite typically the pseudo-halogen when it was exchanged for iodide in the joint cavity gave the same potential as iodide. However, subsequent additions of iodide gave lower potentials, the sign eventually becoming negative and reaching a limit.

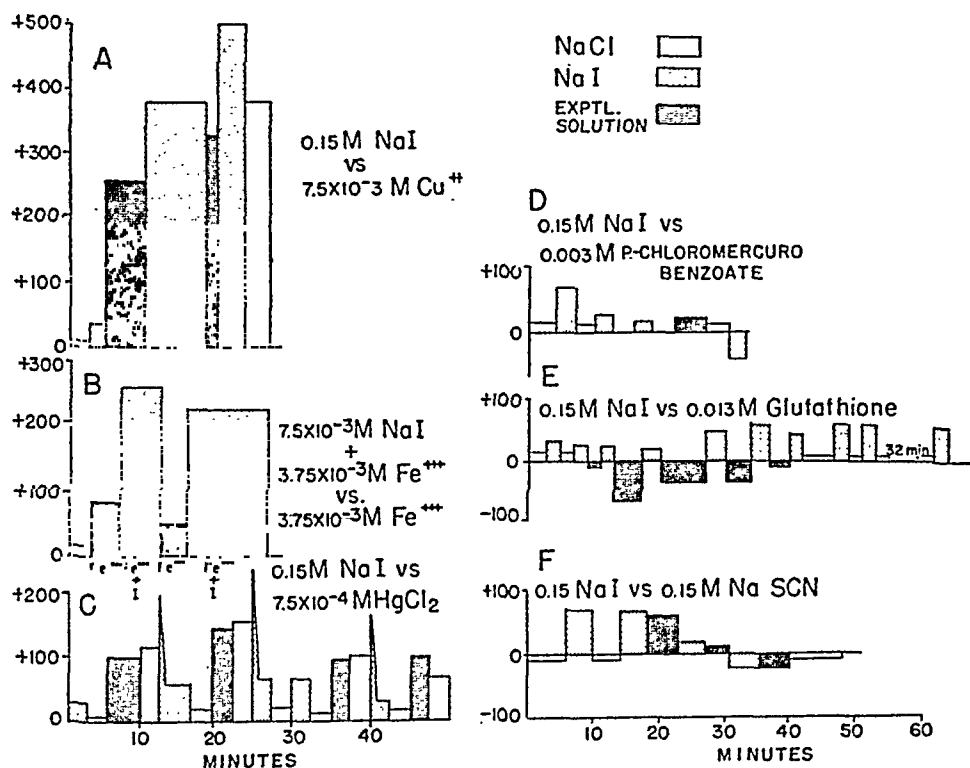


Fig. 2. EFFECTS of inhibitor combinations on membrane potentials. Iodide series.

A detailed tabulation of the effects of the various systems is included in table 1. The systems are grouped into three classes, according to whether the components behave antagonistically, synergistically, or independently. The first class includes pairs of reagents which tend to nullify the potential, while those of the second class enhance the primary potentials. The third class includes systems whose components have no significant effect on the primary potential of the other component.

#### DISCUSSION

The behavior of the NaI-CuCl<sub>2</sub> system described in figures 2A and 3 appears to be of special significance since it results in the highest positive potentials which have thus far been observed. Quite characteristically the alternate application of these inhibitors produced a potential of approximately 500 mv. with the positive sign at the joint cavity, where the solutions were applied. According to Ball (6), this is the

order of magnitude of the difference of standard redox potentials between cytochrome b and cytochrome oxidase.

Electrochemical considerations have previously led to the formulation of the membrane potential in the expression

$$E_{obs} = \bar{E}_{st}^0 - \bar{E}_{st}' \quad (1)$$

where  $E_{obs}$  is the observed membrane potential, while  $\bar{E}_{st}^0$  and  $\bar{E}_{st}'$  denote mean differences of potential between electron source and terminal in the cytochrome system. The former term denotes the standard value for normal tissue, the latter representing the value in the presence of a given inhibitor (2). If the potentials at

TABLE 1

CLASSIFICATION OF SYSTEMS	NO. OF EXPERIMENTS	FIG. NO.
<i>Antagonistic Pairs</i>		
1. $\text{FeCl}_3$ -Na pyrophosphate.....	6	1D
2. Heavy metal-thiols (10 systems).....	30	
3. NaI-p-chloromercurobenzoate.....	3	2D
4. NaI-NaSCN.....	8	2F
5. NaI, $\text{I}_2$ .....	12	
<i>Synergistic Pairs</i>		
6. $\text{FeCl}_3$ -NaCN.....	4	1A
7. NaI-Glutathione.....	8	2E
8. NaI- $\text{CuCl}_2$ .....	6	2A, 3
9. NaI- $\text{FeCl}_3$ .....	3	2B
10. <sup>1</sup> NaI- $\text{HgCl}_2$ .....	3	2C
<i>Independent Pairs</i>		
11. $\text{CuCl}_2$ -Na pyrophosphate.....	4	1E
12. $\text{CuCl}_2$ -Na ascorbate.....	2	

N.B.: The  $\text{CuCl}_2$ -NaCN system has not been classified because of inconsistent results (fig. 1B and C).

<sup>1</sup> Initial transient effect synergistic.

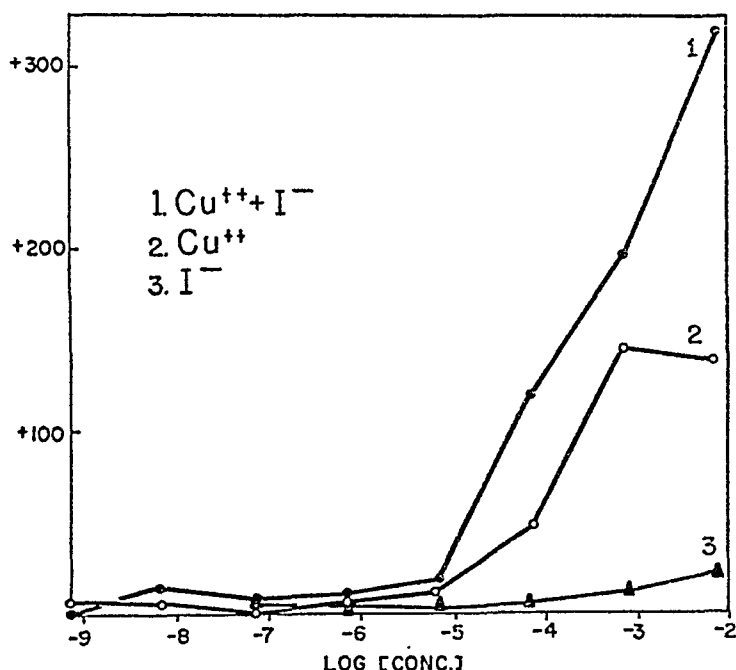
the electron source and terminal are given Ball's values, then  $E_{st}^0$  has a value of 540 mv. This should theoretically represent the limiting observed membrane potential under conditions when  $E_{st}'$  becomes zero, that is to say when the cytochromes and other electron transfer units at the inhibited side of the membrane are all at the same potential. Evidently such a condition occurs in the double inhibition of copper and iodide ions. The oxidation of cytochrome b has been proposed to account for the cupric ion potentials which develop at concentrations above  $10^{-5}$  M (2). The oxidation of iodide by peroxidase, also an electron conducting unit (7), has been postulated to explain the primary iodide potential. Accordingly, the primary mechanisms have been assumed to be the oxidation of cytochrome b and the reduction of a ferric enzyme at a higher potential. The combination of the two inhibitors evidently results in a coupling of two electron transfer units which eventually come to the same

potential by an oxidation reduction reaction. Solutions of cupric iodide have redox potentials of the order of 350 mv., somewhat higher than the standard potential of cytochrome c, and intermediate between cytochrome b and cytochrome oxidase.

Accordingly inhibitors should act synergistically if simultaneously they oxidize the electron acceptor components and reduce the electron donor components of the cytochromes or other electron transport systems, thus tending to bring electron source and terminal to the same potential.

Cyanide is considered to produce inhibition by forming a complex with cytochrome oxidase, the combination having a lower redox potential than the free enzyme (6). This concept has been applied in explaining the membrane potentials produced by cyanide (2). Synergistic effects between ferric ions and cyanide (figure 1A) or between cupric ions and cyanide (figure 1B) are explicable as a simultaneous

Fig. 3. EFFECT of concentration on membrane potential. Cupric iodide system. Ordinate: Potential in mv. Abscissa: Concentration on logarithmic scale.



increase of the source potential from heavy metal oxidation and a decrease of terminal potential by the formation of a cyanide complex. Antagonism between the inhibitors (figure 1C) might be explicable as complex formation between the inhibitors. Such a mechanism would also explain the antagonism between ferric ions and pyrophosphate (figure 1D). The independence of cupric ions and pyrophosphate is consistent with their failure to form complexes (figure 1E).

The observations on  $\text{NaI-HgCl}_2$  require both the above mechanisms for their explanation. The initial 'spike' is evidently explicable in terms of the mechanism given for the synergy of cupric and iodide ions. The secondary antagonism, resulting in a fairly stable potential lower than that of the  $\text{HgCl}_2$  solution, can be explained as the formation of mercurous iodide, which forms a soluble undissociated complex in an excess of iodide.

Additional data relating to the behavior of iodide ion are given in figure 2D, E, F. The sulfhydryl inhibitor, p-chloromercuribenzoate, has been observed to abolish

the potential normally given by iodide, while the sulfhydryl activator, glutathione, has been found to enhance it. Evidently the oxidation of iodide ion depends on the state of enzymatic sulfhydryl groups. Its oxidation may also evidently be affected by ions of a similar chemical nature such as pseudo-halogens. From figure 1F, it is evident that thiocyanate is oxidized at a potential near that of the preceding iodide solution. However, iodide is oxidized at a lower level than that of the preceding thiocyanate. The effects presumably involve positive and negative ion catalysis and possibly free radical chain reactions.

In connection with these considerations it is pertinent to reconsider the effects of the iodide-iodine system and the chloride-iodine system, which were previously studied (1). The latter system gave an initial negative potential, which was interpreted as oxidation at the electron terminal level, i.e. cytochrome oxidase. By contrast, high positive potentials were produced by molecular iodine in the presence of iodide ion. The latter system has a standard redox potential of the order of 500 mv., in the neighborhood of the value proposed for cytochrome oxidase (6). If the iodide ion reduces electron transfer units at high potentials the effect would be to nullify the oxidation of those units by molecular iodine. Oxidation of units of lower redox potential would, however, occur, i.e. at the cytochrome b level, where iodine may be reduced but iodide cannot be oxidized. Accordingly, the potential at the electron source approaches the terminal potential, with a resulting positive potential observed.

#### SUMMARY

The effects of binary combinations of various inhibitors on the membrane potential of the synovialis in dogs have been studied. Synergistic combinations of inhibitors included cupric ions with iodide, ferric ions with iodide, and ferric ions with cyanide. Antagonistic combinations included ferric ions with pyrophosphate, and mercuric ions with iodide. The synergistic combinations involve simultaneous increase of potential of lower cytochromes and decrease of potential at the oxygen terminal. Antagonistic combinations involve complex formations between the inhibitors. The iodide potential is increased by glutathione, a sulfhydryl activator and is decreased by p-chloromercurobenzoate, a sulfhydryl inhibitor.

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# NATURE OF PYROGEN FEVER: EFFECT OF ENVIRONMENTAL TEMPERATURE ON RESPONSE TO TYPHOID-PARATYPHOID VACCINE<sup>1</sup>

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THE onset of pyrogen-induced fevers in man involves simultaneous inhibition of heat loss and stimulation of heat production (1-3). A small part of the increased calorigenesis can be attributed to the van't Hoff effect (4) and slight stimulation of overall metabolism may be involved (5) but shivering is the main cause (1-3).

These and many similar observations are usually explained in terms of the 'thermostatic resetting' theory of fever, first stated clearly by Liebermeister in 1875 (6), which attributes fever to the 'resetting' of the thermostatic centers to a higher operating temperature. This theory provides a succinct description of the altered thermoregulatory status during fever but its implications have not been tested critically and there is no evidence to indicate how such a change in thermal thresholds might be effected.

It was noted (7) that administration of pyrogen to animals exposed to cold caused inhibition of shivering and consequent hypothermia. This result seemed incompatible with the simple thermostatic reset theory, which postulates exaggerated chemical cold defense during fever. The influence of environmental temperature on the thermal and metabolic responses to pyrogens was therefore studied, using oxygen consumption as a measure of calorigenesis.

## METHODS

Mature male New Zealand White rabbits 2.0 to 4.0 kg. in weight were used. A closed circuit metabolism system of about 12 liters in capacity was employed with air circulating at 5 to 7 liters per minute. This was enclosed in a box thermostatically regulated within  $\pm 0.5^\circ$ . The system was kept at atmospheric pressure by replacing the oxygen through automatic water-filled burettes as it was absorbed (8). Thus the animal breathed air of normal composition. The oxygen in the burettes was allowed to come to temperature and pressure equilibrium with room air and to saturation with water vapour before use. Oxygen consumed was read every 5 minutes, but since intermittent activity of the animals and other factors often caused marked variations in apparent oxygen uptake from one 5-minute period to the next, all calculations were based on periods of 50 to 90 (usually 60) minutes. Experimental periods were always compared with immediately preceding control periods of like duration in which the animal was handled similarly, usually with injection of pyrogen-free water. Use of a face mask or of tracheotomy was found to be impractical since even light restraint may profoundly disturb thermoregulation in rabbits (9) and prevent development of typical fevers.

The pyrogen used in most experiments was Cutter Laboratories' typhoid-paratyphoid triple vaccine (TPT) containing about 1000 million *E. typhosa*, 500 million *S. paratyphi* and 500 million

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*S. schottmilleri* organisms per ml. The usual dose was about 0.015 ml/kg., diluted tenfold for injection via the marginal ear vein. This is about 100 times the minimal pyrogenic dose and gives a maximal fever of consistent pattern. Large doses give erratic fevers often accompanied by toxic signs including dyspnoea, hypotension and occasionally death. The results were confirmed using the endogenous pyrogen 'Pyrexin', prepared by Menkin (10) from aseptic pleural exudate. This material is incompletely soluble in water so was finely pulverized then suspended for injection in 1 ml. of pyrogen-free water by repeated passage through a 26-gauge needle.

Results are usually summarized as the mean and standard error of the mean: where scatter is indicated by the standard deviation the letters 'S.D.' appear.

## RESULTS

*Rabbits in Moderate and Warm Environments.* The response of rabbits to moderate doses of TPT shows minor variations but the general pattern is consistent (fig. 1). Seven to 15 minutes after injection, thermal polypnoea is inhibited and vasoconstriction lowers the ear temperature to values near that of the environment, the pulse in the central artery becoming impalpable. The resulting rise in rectal temperature is nearly always biphasic, being interrupted by a plateau or temporary fall after about an hour due to return of ear vasodilation and polypnoea in variable intensity. Renewed inhibition of the heat loss mechanisms develops towards the end of the second hour and maximum temperature (rarely over 42°) is reached during the third hour. Defervescence, marked by polypnoea and strong vasodilation in the ears, begins shortly after the maximum temperature is reached and equilibrium at or near the control temperature is usually attained 4 to 6 hours after injection.

Table 1 summarizes the changes in rectal temperature seen during the first 5 hours of fever in 38 animals given 0.001 to 0.02 ml/kg. of vaccine at 18.5 to 31.0°.

With large doses of vaccine (0.3–0.5 ml/kg.) the early course of fever is similar but defervescence may not be completed for 15 hours or more, the rectal temperature showing erratic fluctuations. Such animals commonly show low maximum temperatures and may develop hypothermia after an initial rise. With very small doses (0.0001–0.001 ml/kg.) the initial temperature rise is as great as with larger amounts but the second rising phase of fever is often absent, final defervescence beginning after 1½ to 2 hours. Thus the chief effect of varying the dosage over a wide range is upon the duration of fever.

At environmental temperatures above about 32° uninjected animals showed gradual rise of body temperature. When the rectal temperature was above 41° at the time of injection, vaccine caused little or no inhibition of heat defense mechanisms.

*Oxygen consumption.* Normal oxygen consumption varied from 7.20 to 14.40 ml/kg/min. (dry gas at S.T.P.), in 41 determinations on 21 animals (mean 10.16, S.D.  $\pm$  1.34). These values agree with the higher values given by various investigators (11, 12) for adult rabbits.

Table 2 summarizes the data obtained from 19 animals given moderate amounts of vaccine and from 3 animals given large amounts. All the former group showed increased oxygen consumption for the first hour following injection. One animal given a large dose showed a similar increase in oxygen uptake but two showed a slight decrease. Control water injections had no effect except in one case (*rabbit 55*) where the water must have been contaminated with pyrogen (temperature rose 1.75° and oxygen consumption 0.9 ml/kg/min.). Mean values for the hour preceding and the hour following water injection in 12 animals were 10.19, S.D.  $\pm$  1.03 and 10.20,

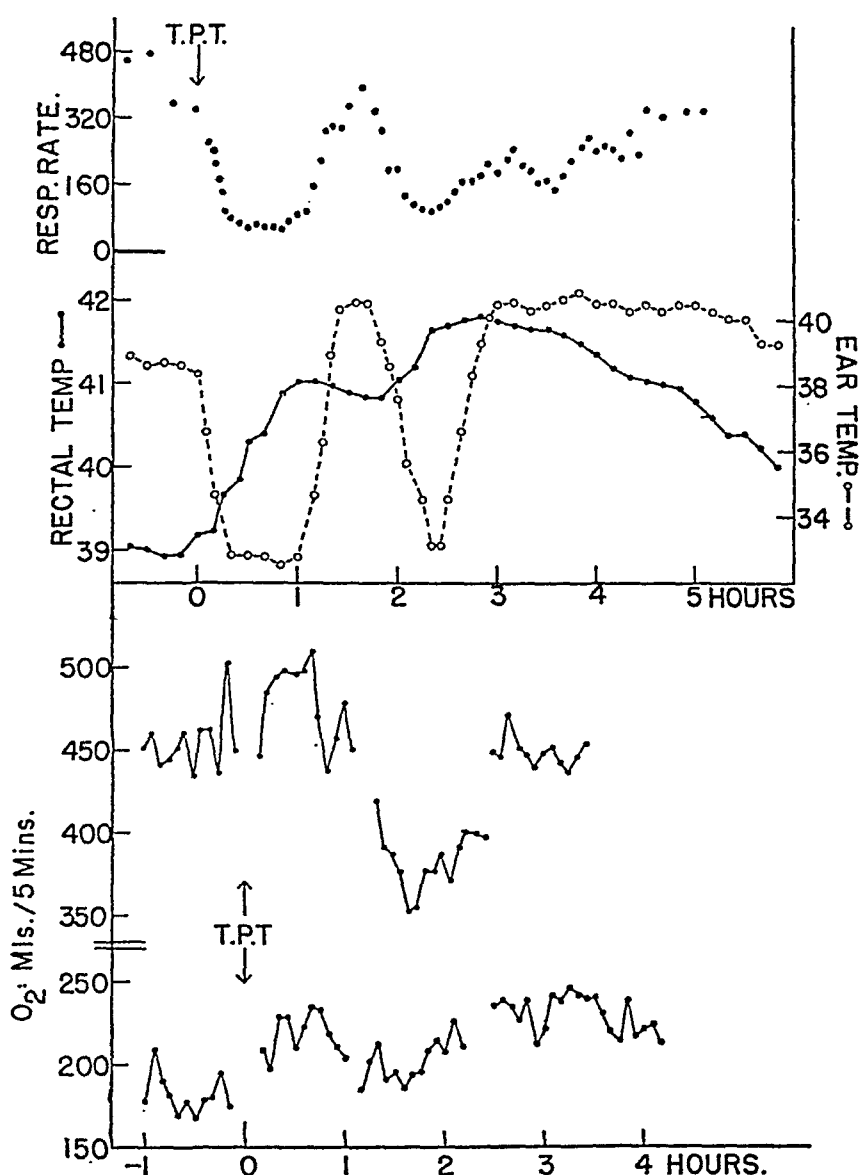


Fig. 1 (upper). CHANGES IN RECTAL TEMPERATURE, ear temperature and respiratory rate in rabbit 193 following injection of TPT at 30° environmental temperature.

Fig. 2 (lower). Upper graph: MEAN OXYGEN CONSUMPTION for successive 5-minute intervals in 8 shorn rabbits exposed to cold and given TPT. Lower graph: Similar data for 8 unshorn rabbits at moderate environmental temperatures.

S.D.  $\pm 0.88$ . The data for animals given moderate doses of vaccine yield the following mean values for oxygen consumption for the hour immediately preceding injection and the first 3 hours of fever:

Control period:	(N = 19)	Mean 9.76, S.D. $\pm 1.80$
1st hour of fever:	(N = 19)	Mean 11.47, S.D. $\pm 1.92$
2nd hour of fever:	(N = 14)	Mean 10.71, S.D. $\pm 2.14$
3rd hour of fever:	(N = 9)	Mean 10.56, S.D. $\pm 1.47$

The mean increase in oxygen consumption for the first hour of fever was  $1.71 \pm 0.20$  (mean and standard error). Corresponding differences from the control level for the second and third hours were  $1.25 \pm 0.24$  and  $1.82 \pm 0.30$ . The mean difference

between the first and second hours of fever was  $-0.83 \pm 0.23$ ; between the second and third hours,  $0.41 \pm 0.31$ . All these differences, except the last, are highly significant statistically. Thus oxygen consumption is increased during the first, second and third hours of fever under the conditions used but is less during the second hour than during the first.

The time course of variations in oxygen uptake is shown in more detail in figure 2 (*lower graph*). This graph was constructed by averaging the raw data from 8 animals for corresponding 5-minute periods. The initial increase in oxygen usage is evident about 20 minutes after injection and is transient, near-normal values being restored towards the end of the first hour. In the individual cases peak values for oxygen consumption occurred 15 to 40 minutes after injection and were more pronounced than is indicated by the composite graph. The initial period of increased oxygen uptake thus corresponds in time to the initial phase of inhibition of heat loss mech-

TABLE 1. CHANGES IN RECTAL TEMPERATURE FOLLOWING VACCINE

	1st HR.	2nd HR.	3rd HR.	4th HR.	5th HR.
Range (°C.)	+0.55	-0.65	-0.50	-1.50	-1.20
	+1.85	+1.15	+0.90	+0.50	-0.40
Mean & standard error	+1.32±0.05	+0.04±0.07	+0.34±0.07	-0.48±0.07	-0.79±0.05
Number showing increase	38	23	26	2	0
Number showing decrease	0	14	6	27	20
Number studied	38	37	32	29	20

anisms. It cannot be regarded as a secondary effect of rising body temperature but must be due to a specific stimulating effect of vaccine.

The second hour of fever includes the plateau phase of reduced or reversed temperature change. Oxygen consumption, although less than during the first hour, remained above the control level in all but one of 14 animals. The stimulation persisted and was usually accentuated during the third hour. In general, changes in oxygen consumption after the first hour of fever parallel changes in rectal temperature and may be attributed mainly to the van't Hoff effect. The data give no suggestion of a continuance or recurrence of a specific stimulating effect of vaccine beyond the first hour. The metabolic cost of sustained, rapid panting during defervescence probably contributes to the high values observed in that phase. When rectal temperature is forced up, in uninjected animals, by exposure to environmental temperatures of 33° to 35°, oxygen consumption increases much more than would be predicted on a basis of a  $Q_{10}$  of 3.0.

There was no parallelism between the amount of the metabolic increase during the first hour of fever and the rise of temperature. The rabbit showing the least increase in oxygen consumption (no. 219) showed a greater rise in rectal temperature during the first hour than that showing the largest increase in oxygen usage (no. 204).

TABLE 2. OXYGEN CONSUMPTION ( $O_2$ ) AND CHANGES IN RECTAL TEMPERATURE ( $\Delta T$ ) FOLLOWING INJECTION OF TPT VACCINE IN RABBITS AT MODERATE ENVIRONMENTAL TEMPERATURES

ANIMAL <sup>1</sup>	BEFORE TPT	AFTER TPT				ENV. TEMP.	DOSE
		1st Hr.	2nd Hr.	3rd Hr.	4th Hr.		
						°C.	ml/kg.
206 $O_2$	7.20	9.56	8.63	9.73	8.95	30.0	0.017
39.3 $\Delta T$	-0.10	+1.45	+0.20	+0.85	-0.35		
197 $O_2$	8.02	10.57	9.63	9.28	9.55	28.0	0.017
39.3 $\Delta T$	+0.20	+1.20	+0.50	+0.45	-0.70		
222 $O_2$	7.59	10.00	9.67	9.68	9.04	30.0	0.017
39.4 $\Delta T$	-0.17	+1.21	+0.06	+0.70	-0.50		
222 $O_2$	7.91	9.34	9.30	9.64		26.0	0.017
39.4 $\Delta T$	-0.30	+0.95	+0.25	+0.70			
221 $O_2$	9.51	11.23	10.81	11.16	10.69	26.0	0.017
39.4 $\Delta T$	-0.07	+1.27	-0.10	+0.68	-0.40		
201 $O_2$	9.97	12.17	12.40	13.15		28.0	0.017
39.7 $\Delta T$	-0.35	+1.50	+1.15	+0.20			
205 $O_2$	10.22 <sup>2</sup>	12.26	11.80	10.50		18.5	0.014
39.2 $\Delta T$	0.00	+0.82	+0.91	+0.30			
181 $O_2$	10.00 <sup>2</sup>	10.81	10.75	12.69	12.13	26.0	0.010
39.0 $\Delta T$	+0.40	+0.70		+0.55			
217 $O_2$	8.22	10.12	8.39	9.22	8.86	31.0	0.017
39.7 $\Delta T$	-0.10	+1.25	-0.13	+0.28	-0.35		
217 $O_2$	10.18 <sup>2</sup>	10.81				20.0	0.014
39.5 $\Delta T$	+0.15	+1.20	+0.50	+0.30	-0.40		
196 $O_2$	8.10 <sup>2</sup>	9.07	8.20			18.5	0.014
39.4 $\Delta T$	-0.02	+1.20					
204 $O_2$	10.02 <sup>2</sup>	13.62	12.41			19.5	0.015
39.1 $\Delta T$	-0.10	+1.15	+0.45	+0.45			
171 $O_2$	10.10 <sup>2</sup>	11.90	9.53			25.5	0.010
39.2 $\Delta T$	+0.25	+1.15					
152 $O_2$	10.30 <sup>2</sup>	12.40				18.5	0.012
39.4 $\Delta T$	+0.10	+1.40					
219 $O_2$	9.95 <sup>2</sup>	9.97				18.5	0.015
39.0 $\Delta T$	-0.03	+1.18	+0.45	+0.45			
208 $O_2$	10.95 <sup>2</sup>	11.60				18.5	0.016
39.7 $\Delta T$	+0.10	+1.05					

TABLE 2.—*Continued*

ANIMAL <sup>1</sup>	BEFORE TPT	AFTER TPT				ENV. TEMP.	DOSE
		1st Hr.	2nd Hr.	3rd Hr.	4th Hr.		
210 O <sub>2</sub>	9.55 <sup>2</sup>	11.45				°C.	ml./kg.
39.9 ΔT	-0.15	+1.55				17.0	0.019
2 O <sub>2</sub>	13.20	14.25	12.00			22.0	0.140
39.5 ΔT	0.00	+1.05	+0.60				
1 O <sub>2</sub>	14.40	16.90	16.20			23.0	0.155
39.5 ΔT	-0.20	+1.60	-0.65				
8 O <sub>2</sub>	11.10 <sup>2</sup>	11.95	11.00			32.5	0.370
39.2 ΔT	+0.20	+2.40					
55 O <sub>2</sub>	11.60 <sup>2</sup>	11.32	13.21	13.48		24.5	0.430
39.2 ΔT	+1.75	+1.95					
57 O <sub>2</sub>	12.50 <sup>2</sup>	12.22	11.40			22.5	0.610
39.5 ΔT	0.00	+0.60	+0.30				

<sup>1</sup> Initial rectal temperature is shown before the symbol ΔT. Temperature changes shown are non-cumulative.

<sup>2</sup> Water-injected control periods.

Environmental conditions were very similar in the two cases. Delcourt-Bernard's data for man (2) show a similar lack of parallelism between the metabolic increase and the temperature rise during the chill phase.

The mean increase in oxygen consumption for the first hour (1.71 ml/kg/min.) corresponds to an increased heat production of about 0.49 Cal/kg/hr. Assuming a specific heat of 0.83 this heat could, if stored, account for a rise in average body temperature of about 0.6°; the mean rise in rectal temperature seen was 1.24°. No attempt was made to calculate changes in average body temperature but changes in rectal temperature suggest that, under moderate environmental conditions, heat storage always exceeds the supplemental amount produced by vaccine. This is certainly so when only minor changes in oxygen usage occur.

The overall pattern of changes in heat production in rabbits given pyrogens is similar to that described for man (1-3) if allowance is made for the more rapid course of fever in rabbits. But in contrast to the very striking increase in oxygen consumption seen during the chill phase in man (over 300% in some cases) the corresponding increase in the rabbit averages only 17.6 per cent. The greatest increase observed for the first hour of fever was 36 per cent. The obvious reason for this difference is the absence of shivering during the onset of fever in the normal rabbit.

Metabolic stimulation by vaccine was undiminished in those rabbits exposed to temperatures at the upper limit of heat tolerance (30-31°). A similar transient increase in oxygen consumption was seen in 4 animals exposed to 32.5° to 34.5° and consequently showing slowly rising rectal temperature prior to injection.

*Rabbits Exposed to Moderate Cold.* In man, febrile chills are usually greatly diminished by warmth and accentuated by cold. It was thought that exposure to cold might bring out chill responses to vaccine injection in rabbits, with consequent increase of the metabolic response. This was not the case.

Table 3 shows the effects of vaccine (0.015 ml/kg.) on rectal temperature and oxygen consumption in 7 rabbits when exposed to  $-2^{\circ}$ . Corresponding data for the same animals at  $17$  to  $20^{\circ}$  are given in table 2. A three-week period separated the two sets of observations to eliminate possible effects of developed refractoriness. The rabbits were exposed to cold for at least an hour before control observations were begun and showed strong activation of the physical cold defense mechanisms (postural adjustments, piloerection, cutaneous vasoconstriction) but only slight shivering. Rectal temperature showed negligible depression (mean  $0.07 \pm 0.1^{\circ}$ ) com-

TABLE 3. OXYGEN CONSUMPTION ( $O_2$ ) AND CHANGES IN RECTAL TEMPERATURE ( $\Delta T$ ) FOLLOWING INJECTION OF TPT VACCINE IN RABBITS AT  $-2^{\circ}$

ANIMAL	BEFORE TPT	AFTER TPT		ANIMAL	BEFORE TPT	AFTER TPT	
		1st Hr.	2nd Hr.			1st Hr.	2nd Hr.
204 $O_2$	16.08	17.03	12.10	210 $O_2$	12.89	14.42	12.17
$\Delta T$	+0.35	+0.72	-0.52	$\Delta T$	+0.25	+1.40	+0.10
205 $O_2$	14.00	15.68	12.94	208 $O_2$	12.96	16.00	13.03
$\Delta T$	+0.14	+1.06	-0.06	$\Delta T$	+0.03	+1.37	0.00
196 $O_2$	9.32	10.99	10.30	217 $O_2$	13.35	14.92	11.96
$\Delta T$	0.00	+0.77	+0.19	$\Delta T$	+0.12	+1.10	+0.08
219 $O_2$	13.47	14.47	12.26				
$\Delta T$	+0.24	+1.00	-0.10				

pared to that observed at moderate temperatures. Oxygen consumption was increased  $3.30 \pm 0.58$  ml/kg/min. or 33.5 per cent.

The mean increase in oxygen consumption for the first hour following vaccine was  $1.39 \pm 0.14$  at  $17^{\circ}$  to  $20^{\circ}$  and  $1.63 \pm 0.26$  at  $-2^{\circ}$ . The apparent slight increase in stimulation in the cold ( $0.24 \pm 0.26$ ) is obviously not significant statistically. The mean rise of rectal temperature one hour after injection (5 animals) was  $1.18$ , S.D.  $\pm 0.26$  at  $17^{\circ}$  to  $20^{\circ}$  and  $1.06$ , S.D.  $\pm 0.24$  at  $-2^{\circ}$ ; mean difference  $0.12 \pm 1.0^{\circ}$ . Thus cooling did not affect the initial temperature rise appreciably.

During the second hour of fever at  $-2^{\circ}$  oxygen consumption appeared to be *reduced* relative to the control level (mean decrease  $-1.04 \pm 0.58$  ml/kg/min.) in contrast to the situation in warm environments. The statistical significance of this depression is questionable ( $P > 0.1$ ) but the data indicate that the inhibition of oxygen uptake seen in severely cooled animals (see below) is already evident in rabbits exposed to  $-2^{\circ}$ .

Figure 3 shows changes in oxygen consumption and rectal temperature in a rabbit exposed to  $0^{\circ}$  and given 0.2 ml/kg. of TPT. The data plotted are for oxygen used in successive 5-minute periods. Following the initial stimulation of metabolism

a depression of oxygen uptake is evident, with subsequent recovery of the control level. Of 6 animals treated similarly, 3 showed hypothermia and depression of oxygen consumption beginning 30 to 50 minutes after injection.

*Shorn Rabbits Exposed to Cold.* To obtain strong stimulation of shivering, rabbits were closely shorn (except for the feet) then exposed to cold ( $-8.0^{\circ}$  to  $+7.0^{\circ}$ ). If exposed immediately after shearing such animals usually showed progressive hypothermia, but if not exposed until the day following shearing adult animals usually maintained constant rectal temperature for many hours although at a slightly depressed level. Shivering was strong and continuous and oxygen uptake markedly increased (240% of normal).

Temperature response to vaccine under these conditions showed much individual variation, the most constant feature being a rapid fall of temperature beginning 40

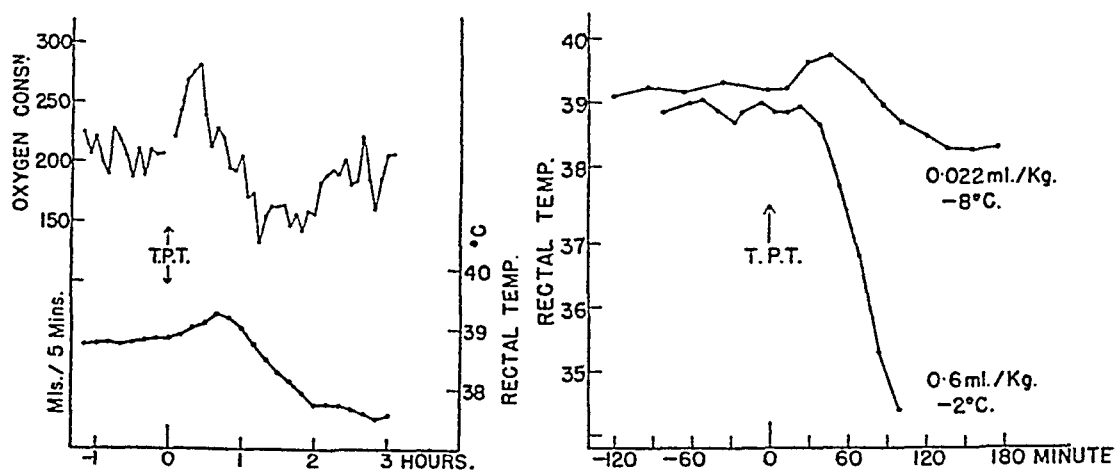


Fig. 3 (left). CHANGES IN RECTAL TEMPERATURE and in oxygen consumption of rabbit following i.v. injection of 0.2 ml/kg. of TPT at environmental temperature of  $0^{\circ}$ .

Fig. 4 (right). CHANGES IN RECTAL TEMPERATURE following TPT injection in 2 shorn rabbits exposed to cold.

to 60 minutes after injection and carrying the animal into decided hypothermia. In 12 of 29 cases the fall in temperature was preceded by slight fever (maximum rise  $1.0^{\circ}$ ); in only 3 cases temperature did not fall below the control level. Figure 4 shows two types of reaction encountered. Time of onset of the fall in temperature varied from 25 to 90 minutes after injection but the delay was rarely over 60 minutes. Large doses of vaccine hastened the onset of cooling and increased its severity. Subsequent behavior of the animal seemed to depend mainly on the degree of hypothermia developed by the end of the second hour; if moderate, the temperature increased again in the third hour, but if hypothermia were severe the temperature either continued to fall or became relatively stable at the depressed level. When removed to a warm environment normal temperature was regained rapidly in all cases.

No increase in ear temperature or in respiratory rate could be detected at the time of onset of the cooling phase but shivering was obviously diminished and sometimes abolished. The fall in temperature thus seemed to be due entirely to failure of the main mechanism of cold-stimulated calorogenesis.

Table 4 shows changes in oxygen consumption and in rectal temperature in 8 shorn animals given small amounts of vaccine at environmental temperatures from  $-1.0^{\circ}$  to  $7.0^{\circ}$ ; data for two of the same animals when given large amounts of vaccine are also shown. Corresponding data for most of these animals when run at moderate

TABLE 4. OXYGEN CONSUMPTION ( $O_2$ ) AND CHANGES IN RECTAL TEMPERATURE ( $\Delta T$ ) FOLLOWING INJECTION OF TPT VACCINE IN SHORN RABBITS EXPOSED TO COLD

ANIMAL <sup>1</sup>	BEFORE TPT	AFTER TPT			ENV. TEMP.	DOSE
		1st Hr.	2nd Hr.	3rd Hr.		
204 $O_2$	22.41	23.86	22.02	22.40	$^{\circ}C.$	ml/kg.
38.6 $\Delta T$	+0.13	+0.50	+0.05	+0.32	2.0	0.016
205 $O_2$	22.03	23.80	20.26	23.43	5.0	0.025
39.1 $\Delta T$	+0.02	+0.48	-0.82	-0.08		
196 $O_2$	22.31	22.86	19.04	15.91	-1.0	0.026
38.3 $\Delta T$	-0.23	+0.28	-1.12	-2.98		
219 $O_2$	24.07	23.33	20.79	23.93	2.0	0.030
38.6 $\Delta T$	-0.01	+0.04	-1.13	+0.10		
208 $O_2$	27.56	29.23	27.07	27.94	-1.0	0.031
39.5 $\Delta T$	-0.14	+0.51	-0.10	+0.06		
217 $O_2$	20.70	21.84	19.08	21.98	7.0	0.026
39.0 $\Delta T$	-0.02	+0.12	-0.12	+0.30		
189 $O_2$	22.22	23.23	11.30		4.0	0.017
39.4 $\Delta T$	-0.03	+0.51	-3.53			
102 $O_2$	22.63	23.76	20.22	21.71	0.0	0.031
39.2 $\Delta T$	-0.30	+0.34	-1.42	-0.35		
204 $O_2$	20.58	20.90	13.82		2.0	0.290
39.5 $\Delta T$	-0.24	-0.47	-2.01			
189 $O_2$	22.37	24.34	16.40	22.99	2.0	0.330
39.3 $\Delta T$	-0.03	+0.65	-1.82	+0.72		

<sup>1</sup> Initial rectal temperature is shown before the symbol  $\Delta T$ .

temperature and at  $-2^{\circ}$  (unshorn) are shown in tables 2 and 3. Rectal temperatures at the end of the control period (i.e. after at least 2 hours' exposure to cold) were depressed an average of  $0.30^{\circ} \pm 0.14^{\circ}$  and fairly stable. Mean increase in oxygen consumption was  $13.60 \pm 0.72$  ml/kg/min. (140%) over the normal.

The mean increase in temperature one hour after injection was  $0.30^{\circ} \pm 0.03^{\circ}$ : only one animal showed a fall of temperature for this period, following a large dose of vaccine. This increase is much less than that shown by the same animals at mod-

erate temperatures (mean difference  $0.78^{\circ} \pm 0.13^{\circ}$ ). The mean drop in temperature for the second hour was  $1.20^{\circ} \pm 0.34^{\circ}$ .

*Oxygen consumption.* In 9 of the 10 runs oxygen consumption showed an increase for the first hour following TPT (mean increase  $1.03 \pm 0.26$  ml/kg/min.). Corresponding differences between the control period and the second and third hours were  $-3.69 \pm 1.04$  and  $-0.47 \pm 0.89$  ml. The mean decrease in oxygen usage between the first and second hours was  $4.72 \pm 1.03$  and the increase between the second and third hours  $1.93 \pm 0.99$  ml. In all 10 cases oxygen consumption for the second hour after injection was less than that during the pre-injection period; it remained below the control level in 4 of 8 animals followed for a third hour. Comparison of the increases observed in the first hour of 'fever' under these conditions with the corresponding increases shown by the same animals at moderate temperatures shows no significant difference (mean difference in stimulation  $0.85 \pm 0.54$  ml., the warm animals showing the greater mean increase).

In figure 2 changes in oxygen consumption in cold shorn animals are compared with corresponding changes in animals at moderate temperatures. The graphs were constructed by averaging the data obtained from 8 animals when exposed to moderate temperatures (*lower graph*) and when exposed to cold after shearing. The increase in metabolism at the onset of fever was of similar magnitude and duration in the two groups, despite the wide difference in control levels. In the cold group the decline in oxygen uptake continued until frank inhibition of cold-stimulated calorigenesis had developed (compare fig. 3 for a single unshorn animal exposed to cold).

*Shorn Cold Rabbits Given 'Pyrexin'.* Although 50 times the dose of vaccine used may be well tolerated by rabbits it seemed possible that inhibition of shivering might be due to toxic materials distinct from the pyrogen in the unfractionated vaccine. The experiments on cold shorn animals were therefore repeated using a non-bacterial pyrogen, 'Pyrexin', prepared from sterile pleural exudate by Menkin (10).

At normal environmental temperatures, intravenous injection of 1.25 to 2.50 mg/kg. of Pyrexin suspended in 1.0 ml. of sterile pyrogen-free water caused a fever essentially identical with that resulting from injection of moderate amounts of TPT, except that defervescence was somewhat delayed, suggesting that this amount of Pyrexin represents a higher dose of pyrogen. Figure 5 shows the febrile response to Pyrexin in the same animal whose response to TPT is shown in figure 1.

Table 5 shows effects of Pyrexin on oxygen consumption and rectal temperature of 4 shorn rabbits exposed to  $0^{\circ}$ . The responses of the same rabbits to TPT under similar conditions are shown in table 4. The response to the two pyrogens is closely similar but the depression of oxygen usage in the second hour of fever is probably more marked with Pyrexin.

#### DISCUSSION

The chief difference between the typical reaction to pyrogens of rabbits exposed to moderate temperatures and that described for man, cat and dog is the lack of perceptible shivering in the rabbit. Accordingly, the increase in oxygen consumption is slight, and fever results mainly from restriction of heat loss. Hildebrandt (13) found no evidence of specific metabolic stimulation following pyrogen injections in

rabbits. At low environmental temperatures, when heat loss is already restricted, the metabolic stimulation may well be essential to the development of fever but it is no greater than at higher temperatures. The difference between the rabbit and other species is probably not fundamental. Thyroidectomized rabbits often shiver following pyrogen injections (14), probably because their basal body temperature is depressed. In man, 'attenuated' reactions to pyrogens that are unaccompanied by chills are often seen, especially when repeated injections have developed partial refractoriness (2) or when the environmental temperature is high (3).

Shivering is clearly an unessential component of the febrile reaction although it promotes rapid rise of temperature. Much of the temperature rise occurs after shivering has ceased and the degree of fever developed is not strikingly less when

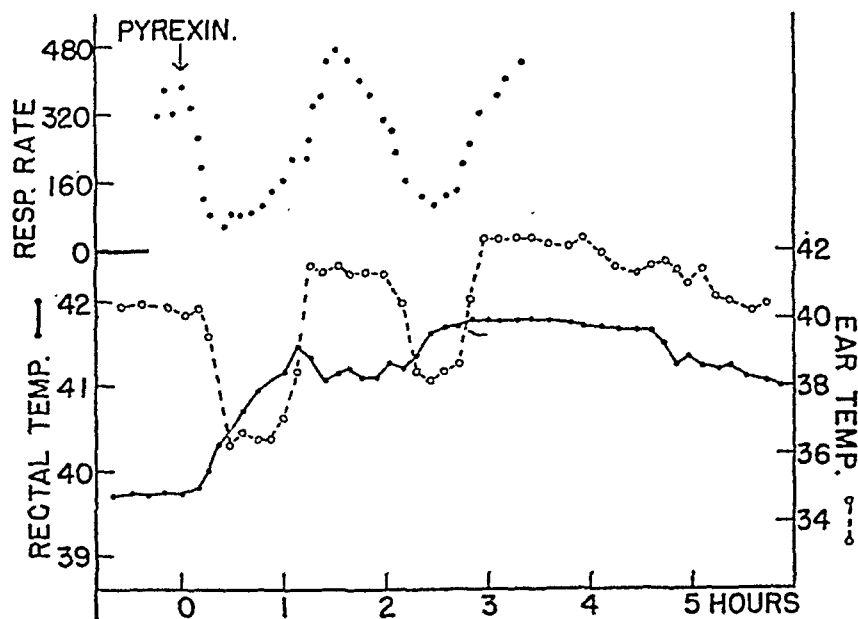


Fig. 5. CHANGES IN RECTAL TEMPERATURE, ear temperature and respiratory rate of rabbit 193 following injection of 2.50 mg/kg. of Pyrexin at 30° environmental temperature (cf. fig. 1).

chills do not occur. In dogs, Wells and Rall (15) found that curarization prior to pyrogen injection caused little diminution in fever.

In terms of the 'thermostatic resetting' theory of fever, the most acceptable explanation of the omission of chills in man is that, in such reactions, the temperature differential between the altered 'set level' and the temperature of the thermostatic nuclei remains small and so excites only physical cold defense. In hot environments this could be so because restriction of heat loss causes a rapid rise in temperature. In partially refractory individuals a more gradual process of resetting may occur, permitting restriction of heat loss to raise the body temperature as fast as the set level is rising. But it is questionable whether this hypothesis can explain the absence of shivering in the pyrogen-treated rabbit. Exposure to cold stimulates strong shivering in rabbits with little fall in rectal temperature; oxygen consumption may be increased more than 150 per cent in this way. The behavior of the heat loss mechanisms following pyrogen injection suggests that the thermal thresholds for activation of these are raised 1.5° to 2.0° within a few minutes after the onset of febrile reactions,

for it is only when this degree of hyperthermia has been induced by exposure to heat that pyrogens fail to inhibit panting or to cause vasoconstriction in the ears. Similar elevation of the shivering threshold should cause strong shivering. The slight and transient stimulation of metabolism that follows pyrogen injection is unaffected by environmental temperature and persists unchanged in the presence of either moderate hyperthermia or moderate hypothermia induced by extreme environmental conditions. Thus it is probable that the increased oxygen uptake is not due to an undetected stimulation or potentiation of shivering but to tissues or mechanisms not involved in the normal defense against cold. Laszlo and Wachstein (16) obtained some evidence suggesting that the heat production of the liver may be increased early in pyrogen fever, but Hall, Fishgold and Grant (17) found that the oxygen uptake *in vitro* of liver slices taken from rabbits 20 minutes after vaccine injection is less than

TABLE 5. OXYGEN CONSUMPTION ( $O_2$ ) AND CHANGES IN RECTAL TEMPERATURE ( $\Delta T$ ) FOLLOWING INJECTION OF PYREXIN IN SHORN RABBITS EXPOSED TO COLD

ANIMAL <sup>1</sup>	BEFORE PYREXIN	AFTER PYREXIN			ENV. TEMP.	DOSE
		1st Hr.	2nd Hr.	3rd Hr.		
205 $O_2$	22.60	20.63	15.02	15.18	°C.	mg/kg.
39.1 $\Delta T$	-0.30	-0.95	-2.40	-1.40	3.0	2.50
196 $O_2$	22.20	22.02	17.14	20.67	0.0	2.50
39.1 $\Delta T$	-0.08	+0.35	-1.77	+0.44		
189 $O_2$	25.09	27.03	19.64	23.87	-2.0	2.50
39.3 $\Delta T$	+0.07	+1.07	-1.64	+0.20		
217 $O_2$	18.03	18.41	15.51	17.65	-3.0	1.25
39.2 $\Delta T$	+0.05	+0.52	-0.42	+0.18		

<sup>1</sup> Initial rectal temperature is shown before the symbol  $\Delta T$ .

that of normal liver. If liver metabolism is increased by pyrogen this stimulation must be due to factors no longer operative in the excised tissue. The immediate cause of the specific calorogenic response to pyrogens in rabbits thus remains obscure. The possibility that endocrine factors such as epinephrine secretion (5) are involved merits study.

The sharp reduction in oxygen consumption beginning about an hour after vaccine injection in animals exposed to cold is due to reduction or abolition of shivering. Although oxygen consumption remains above the control level, some inhibition of metabolism may occur in the non-shivering animal at this time, for the expected increase in oxygen consumption due to increased body temperature is not fully realized. Inhibition of shivering can be brought about by many agents and is not necessarily due to action on the thermoregulatory centers. But there is good reason to believe that inhibition of shivering by pyrogens is due to a central action since the phase of inhibition corresponds in time to the transitory phase of renewed ac-

tivity of heat loss mechanisms in animals in warm environments. No increase in respiratory rate or in ear temperature occurred in the cold.

Inhibition of shivering is clearly contrary to the postulate that the thermal threshold of the thermoregulatory centers is raised at this time. It occurs in 'febrile' animals whose body temperatures are still sub-normal due to exposure to cold, and may persist until they have become severely hypothermic. The inhibitory phase is transitory; if gross hypothermia has not developed shivering is resumed after about an hour.

Ranson (18) found that TPT vaccine fever in cats is cyclic in nature, involving alternating activity of heat conservation and heat dissipation mechanisms. When given to cats exposed to severe cold vaccine causes delayed development of hypothermia as in the rabbit (9). It is well known that vaccine injections produce many 'side reactions' (headache, vomiting, hypotension etc.) indicative of disturbed autonomic function and unrelated to temperature control. Recent studies with highly purified bacterial polysaccharides (19) indicate that these exceedingly active pyrogens, rather than contaminants present in the crude vaccine, are responsible for the many functional disturbances that accompany pyrogen fevers. The fact that two pyrogens as widely different in origin as TPT vaccine and 'Pyrexin' produce a virtually identical sequence of reactions is further evidence that the pyrogens themselves are responsible for both thermogenic and thermolytic phases of response. Ranson *et al.* (20) and Chambers and Windle (21) obtained fever following pyrogen injections in cats with lesions of the hypothalamus that had largely destroyed normal thermostatic reactions, and Windle (22) has induced chills and fever in cats with a partially purified pyrogen after blocking the arterial supply to the anterior brain stem.

Thus the concept of 'upward resetting' of the thermal thresholds of the temperature regulating centers can be applied only to certain aspects of the thermoregulatory derangement produced by pyrogens. Other aspects require assumptions directly contrary to this hypothesis. There is, in fact, no satisfactory evidence that the pyrogens do influence the hypothalamic thermoregulatory centers; the scant evidence now available suggests that they may influence thermoregulation by interfering with the motor mechanism of regulation at lower levels in the brain stem rather than by altering the thermal sensitivity of the primary centers.

#### SUMMARY

In rabbits, the body temperature rise following intravenous injection of typhoid-paratyphoid vaccine or of 'Pyrexin' at moderate environmental temperatures is biphasic. Phases of rising temperature are associated with inhibition of heat loss mechanisms and the interpolated 'plateau' with some activity of these mechanisms.

A transient specific stimulation of oxygen uptake accompanies the initial rising phase. This is maximal 15 to 40 minutes after injection and causes a mean increase in oxygen consumption of  $1.71 \pm 0.20$  ml/kg/minute (17.6%) for the first hour of fever. The amount of increase in oxygen consumption for the first hour after injection is unaffected by exposure to warmth (31°), exposure to cold (-2°), or exposure to cold after shearing. In shorn cold animals, marked inhibition of shivering and of

oxygen consumption during the second hour after injection of vaccine or Pyrexin results in hypothermia.

The bearing of these results on current theories regarding the mechanism of fever is discussed.

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# EFFECT OF PREFRONTAL BRAIN LESIONS ON CORRECT CONDITIONED DIFFERENTIAL RESPONSES IN DOGS

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**T**HIS seventh study on the function of the association cells of the cerebral cortex is concerned with an attempt to localize an area of the prefrontal cortex which is essential for correct conditioned differential responses of the foreleg from olfactory stimulation. It was shown previously (1) that prefrontal lobectomy prevented correct olfactory conditioned differential reflexes for hundreds of tests, while like responses from auditory and tactile stimulations (2) were not affected by this lesion. The stimuli used for the positive and negative conditioned reflexes were: 1) olfactory, inhalation of clove oil and asafetida vapors; 2) auditory, tapping a bell and iron cup once per second; 3) tactile, stroking the side of the back with a brush once per second, with and against the grain. Electric shock of the foreleg served as the unconditioned stimulus for developing the positive conditioned reflex, while the command 'No!' served likewise for the negative conditioned reflex. All of the dogs understood the commands 'No' and 'Quiet.' To be correct a positive conditioned reflex must appear within 7 seconds and the foreleg response must be withheld for 10 to 15 seconds for a correct negative conditioned test, depending on the time of the positive conditioned reflexes. The first and usual daily session of tests consisted of 25 trials with the positive conditioned stimulus and 13 with the negative conditioned stimulus. Occasionally these numbers were reversed and the sequence of tests was changed.

## CONTROLS

All tests were made in rooms that were practically sound proof and sounds from within were masked by the continuous running of a motor and air compressor. All dogs were blindfolded and the sensory hairs about the face were cut. In the olfactory tests the inhalations were from clean bottles presented with clean hands about 4 to 10 cm. from the nostrils. Frequent control tests were made with the same bottles closed with glass stoppers or with an empty bottle attached to a long iron wire handle. Occasionally the stimulating vapors were given after the nostrils had been plugged with cotton and taped. To prevent variations of loudness and differences of brush pressure from evoking conditioned reflexes, the strength of the auditory and tactile stimuli were varied in some tests or in the same test, but ordinarily the strength of the stimuli was kept as near constant as possible.

The lesions were produced by suction and the first tests were recorded 3 to 6 weeks after the operation, depending on the extent of cortical destruction. It was

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most important to keep the animals at perfect ease in all tests in which the lesion prevented correct conditioned differential responses. This was especially important in making a long series of negative conditioned tests. As a matter of routine the author always fed and took care of his dogs. They were always petted before and after a session of tests. If they showed signs of anxiety tension, such as licking the face, a panting respiration, abnormal muscular tremor, or a tendency to stretch out or become limp in the harness, the usual 2-minute interval between tests was increased and they were often petted between tests, but never immediately before or directly after a test. Under these precautions the dogs appeared to enjoy the tests in spite of their inability to make correct differential responses.

#### *Lesion A (Fig. 1, Les. A)*

This extirpation was undertaken with 3 dogs and their formalin-prepared brains disclosed on each side of the median line a pit, 6 mm. long by 5 mm. wide, in front of the cruciate sulcus. The area of destruction was slightly wider and extended a little farther caudad for *dog 2*. Marchi-stained sections through the medulla of these dogs revealed slight to considerable degeneration in the pyramids, being most pronounced in *dog 2* and in the right pyramid of *dog 3*. *Dogs 2* and *3* exhibited motor symptoms in the hind limbs during the first days following the operations.

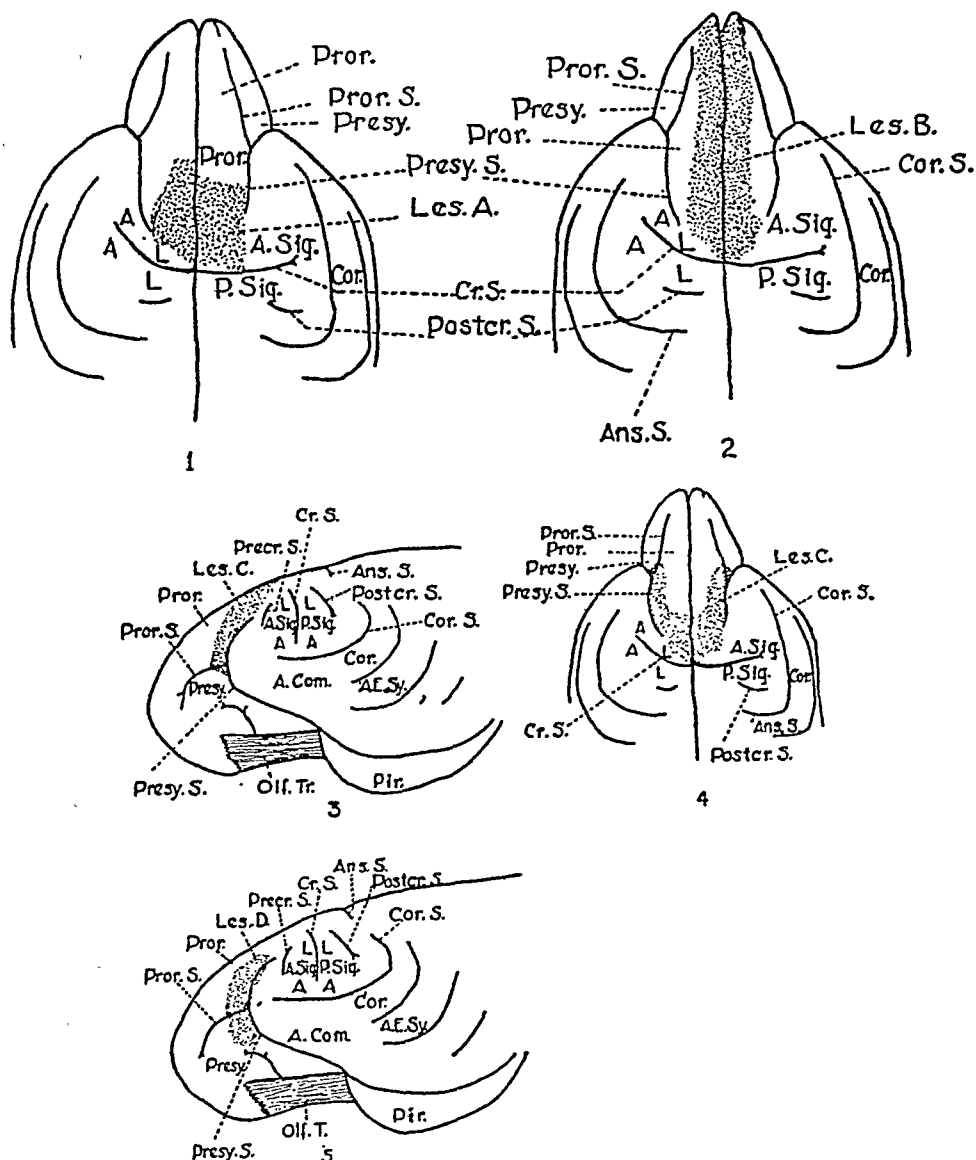
*Results.* The positive olfactory conditioned reflex was established during the first session of tests following the operations with *dogs 1* and *3*, while *dog 2*, having a more extensive extirpation, required 4 sessions of tests for re-establishment of the reflex. The more pronounced effects of the lesion for this dog can readily be attributed to additional injury to contralateral foreleg motor cortex.

Column 3 of table 1 reveals that *dogs 1* and *3* were able to make correct conditioned differential foreleg responses from olfactory stimulation during the first session of tests which followed the operations, while *dog 2*, having a wider and more caudal extirpation, required an additional session of tests. The summary of all tests (columns 6 and 7) demonstrate difficulties that *dog 2* had in acquiring these correct responses. Columns 4 and 5 of the table show that all of the lesion A dogs were ultimately able to make perfect scores for daily sessions of 38 tests. They were also able to respond correctly to a series of alternated positive and negative olfactory conditioned tests when the intervals between tests were of 3 seconds' duration.

#### *Lesion B (Fig. 2, Les. B)*

Formalin-prepared brains from 2 dogs showed this extirpation to have removed a 3 mm. median strip of cortex from each hemisphere, extending from the cruciate sulcus to the olfactory bulbs. No noteworthy degeneration was found in the pyramids of the medulla and the limbs did not exhibit motor symptoms.

*Results.* Two weeks after the operation the first session of 25 tests for the positive conditioned olfactory reflex in both dogs resulted in but one failure to respond and that was for the first trial. Column 3 of table 1 discloses that lesion B produced no effect on correct conditioned differential reflexes from olfactory stimulation and both dogs were able (columns 4 and 5) to make perfect scores for daily sessions of 38 tests. They could also make correct responses for a series of alternated positive and



Figs. 1 to 5. LATERAL AND DORSAL VIEWS of prefrontal lobe of a dog's brain to show exact position of lesions. *Abbreviations:* A. foreleg motor cortex; A. Com., anterior composite gyrus; A.E.Sy., anterior ectosylvian gyrus; Ans. S., ansate sulcus; A. Sig., anterior sigmoid gyrus; Les. A, Les. B, Les. C, Les. D, Lesions A, B, C and D; Olf. Tr., olfactory tract (bulb not shown); Pir. piriform lobe; Poster. S., postcruciate sulcus; Precr. S., precruciate sulcus; Presy., presylvian gyrus; Presys. S., presylvian sulcus; Pror., proreus gyrus; Pror. S., proreus sulcus; P. Sig., posterior sigmoid gyrus.

negative olfactory conditioned tests when the intervals between tests were of 3 seconds' duration.

#### *Lesion C (Figs. 3 and 4, Les. C)*

This U-shaped extirpation was made on the brains of 4 dogs. A dorsal view (fig. 4) shows the base of the U-shaped pit facing the cruciate sulci (Cr. S.) with both arms extending cephalad along the midline and the inner border of the presylvian sulci (Presy. S.) to terminate at the level and a little below the level of the proreus sulci (fig. 3, Pror. S.). The width of the base of these extirpations at the midline ranged from 6 to 7 mm., while the width of the arm portion, varied from 3 to 4 mm. None of these dogs exhibited motor symptoms and there was but little degeneration in the pyramids of the medulla.

*Results.* The positive olfactory conditioned reflex returned with the first trial after the operation for *dogs 8* and *8a* and with the 9th and 11th trials for *dogs 7* and *6*. It should be mentioned that *dog 7* was tested previously for the auditory positive conditioned reflex and that 7 trials were required to evoke this reflex.

It is apparent from columns 3 and 8 of table 1 that none of the lesion C dogs were able to make correct conditioned differential foreleg responses from olfactory

TABLE 1. OLFACTORY-CONDITIONED STIMULI: OIL OF CLOVES AND ASAFETIDA

CORTICAL LESIONS, DOG NO.	BEFORE OPER. DIFF. FIRST APPEARED SESSION NO.	AFTER OPER., DIFF. FIRST APPEARED SESSION NO.	DAILY SESSIONS AFTER DIFF. WAS STANDARDIZED		TOTAL NO. OF TESTS		CHARACTER OF CORRECT COND. DIFF. RESPONSES
			Pos. C-I	Neg. I-C	Pos. C-I	Neg. I-C	
A	1	1st	24-1	0-13	72-4	3-63	Good
			13-0	1-24			
	2	1st	25-0	0-13	315-23	19-168	Good
			13-0	0-25			
B	3	1st	25-0	0-13	101-0	3-92	Good
			13-0	1-24			
	4	1st	25-0	0-13	87-1	3-62	Good
			13-0	0-25			
C	5	1st	25-0	0-13	161-2	4-101	Good
			13-0	1-24			
	6	1st	25-0	13-0	2294-52	1596-107	No
			13-0	24-1			
D	7	1st	25-0	13-0	3054-69	1982-41	No
			13-0	25-0			
	8	1st	25-0	13-0	3106-61	1975-37	No
			13-0	25-0			
	9	1st	25-0	13-0	3280-68	2063-42	No
			13-0	25-0			
	10	1st	24-1	13-0	3037-166	2040-113	No
			13-0	25-0			

Diff. = correct conditioned differential response; Pos. = positive conditioned reflex; Neg. = negative conditioned reflex; Oper. = operations; No. = number; C-I = ratio of correct to incorrect responses; I-C = ratio of incorrect to correct responses.

stimulation. The total number of tests recorded were as follows: *Dog 6* received 4199 trials; *dog 7*, 5146; *dog 8*, 5179. Each dog was allowed at least 3 weeks to recover from the operations and the tests continued for 5 to 6 months. Columns 4 and 5 of the table contain the records of the last two daily sessions for each dog, from which it will be seen that there were no errors for the positive olfactory conditioned tests and only one correct test for the negative olfactory conditioned reflex. Column 7 of the table discloses a few absences of response for the negative olfactory conditioned tests, but column 6 shows as many or more absences of response for the positive conditioned tests.

After completion of 5146 olfactory differential tests for *dog 7*, this dog was tested on 4 successive days for a total of 105 trials with the negative olfactory conditioned reflex alone, with the result that there were 96 foreleg responses and 9 without response. Following these tests with the negative conditioned stimulus, a session of mixed tests resulted in but one absence of foreleg response and that was for a positive conditioned stimulus.

There appears to be no valid reason for allowing more than 3 weeks for recovery from the operations. However, no tests were made on *dog 8a* until 4 months had elapsed. Since the records for this dog do not appear in the table, it can be recorded that this dog was able to make a normal positive olfactory conditioned reflex during the first tests, but there were no signs of correct olfactory conditioned differential responses during 310 tests.

A retesting of the lesion C dogs after the operations for correct conditioned differential responses from auditory stimuli demonstrated that they were all able to make correct responses. Also all of the lesion C dogs acquired correct conditioned differential responses from tactile stimulation with no more difficulty than normal dogs. Furthermore, throughout the entire time during which correct conditioned differential responses could not be elicited from olfactory stimulation, all of the lesion C dogs were not only able to evoke these responses from auditory and tactile stimulation, but they could make correct foreleg responses for a series of alternated positive and negative conditioned tests when the interval between tests was 3 seconds.

#### *Lesion D (Fig. 5, Les. D)*

Formalin-prepared brains from 2 dogs (*9* and *10*), in which this extirpation had been made, revealed identical cortical damage and the possibility of additional injury to the left foreleg motor cortex of *dog 10*. A comparison of figure 5 with figure 3 shows lesion D to be very similar to lesion C, differing from it only by failure to reach the midline, dorsally, by 3 mm. and extending about 3 mm. further ventrad. There was some Marchi degeneration in the right pyramid of medulla sections of *dog 10*, but none in the left pyramid or either pyramid of *dog 9*. Cutaneous placing reflexes could be elicited from the forelegs of both dogs and there were no motor symptoms.

*Results.* Three weeks after the operation, the positive olfactory and the positive auditory conditioned reflexes returned early during the first session of tests with both dogs. Columns 3 and 8 of table 1 show that both lesion D dogs were unable to make correct foreleg conditioned differential responses from olfactory stimulation during 5453 and 5356 trials, which continued over periods of about 6 months. *Dog 10* was allowed a vacation of a month before the last thousand tests were made. It is apparent from the summaries of all tests (columns 6 and 7 of the table) that nearly all of the positive and negative olfactory conditioned tests resulted in foreleg responses, which signifies that the positive tests were nearly all correct and the negative tests were largely errors. The scores for the last two daily sessions (columns 4 and 5) are in complete agreement with the total results.

After completion of the long series of olfactory conditioned differential tests, *dog 10* was tested for 12 daily sessions for the negative conditioned reflex alone, with the result that there were 296 foreleg responses and 4 absences of response.

Both lesion D dogs re-established correct conditioned differential foreleg responses from auditory stimulation during the early tests of one session that followed the operation, and they acquired these responses from tactile stimulation with no more difficulty than an unoperated dog. During the entire period following the operations in which *dogs 9* and *10* were unable to make correct conditioned differential foreleg responses from olfactory stimulation, they were not only able to make these correct responses from auditory and tactile stimulation, but could respond correctly to a series of alternated positive and negative conditioned tests from auditory or tactile stimulation when the interval between trials was of 3 seconds' duration.

#### DISCUSSION

According to Pavlov, Babkin (3) reported that a little more than prefrontal lobectomy in dogs permitted re-establishment of a positive conditioned reflex from auditory and optic stimulation and from tactile stimulation of the extremities, but not from trunk stimulation. However, a negative conditioned reflex from tactile stimulation could always be obtained from any part of the skin. These animals displayed marked hyperthesia and temporary postural disturbances.

Lesions A, B, C or D and the previously reported prefrontal lobectomies (1, 2) sometimes abolished for short intervals the positive conditioned reflex from olfactory, auditory or tactile stimulation, but these temporary effects were attributed to slight injury to the contralateral foreleg motor cortex. More important, this study has shown: 1) that lesions A or B produced no permanent effect on correct conditioned differential responses of the foreleg from olfactory auditory or tactile stimulation and 2) that lesions C or D, while having little or no effect on correct conditioned differential responses from auditory or tactile stimulation, abolished these responses from olfactory stimulation.

The principal effect of lesions C or D was inability to withhold the foreleg response during a negative conditioned test or lack of correct inhibition. However, an occasional negative conditioned test resulted in absence of foreleg response. The total number of these absences of response was in direct proportion to the number of errors for the positive conditioned stimulus. This proportion varied a little according to the number and order of the stimuli used and whether the dog belonged to the easily inhibited or easily excited type, but even in a series of several hundred negative conditioned tests, there were very few absences of foreleg response. At times punishment of errors for the negative conditioned tests appeared to elicit sufficient external inhibition to delay the appearance of the foreleg response for 2 or 3 seconds.

Since lesions C or D did not injure the lateral olfactory tract, it is apparent that the circuit required for making correct conditioned differential responses from olfactory stimulation was severed in the region of the lesion.

It is apparent that lesion C or D destroyed a large portion of Tower's (4) prefrontal inhibitory field for the cat, the stimulation of which was said to arrest movements elicited from stimulation of cortical area 4. It also included all of Garol's (5) and Beckett and Gellhorn's (6) suppressor band 8s for the cat. This suppressor band is physiologically comparable to the other cerebral suppressor bands, which Hines

(7, 8), Dusser de Barenne and McCulloch (9), and Verhaart and Kennard (10) found inhibited movement in monkeys. It should however be noted that Olson (11), with dogs, and Clark and Ward (12, 13), with monkeys, were unable to inhibit movement, other than respiration from stimulation of the prefrontal lobe.

If this prefrontal region is a genuine inhibitory area for movement as the weight of evidence suggests, this study indicates that lesions C or D destroyed the important efferent mechanism for evoking correct conditioned differential responses from olfactory stimulation. To speculate further, one may assume that this prefrontal area developed from a need for inhibition to oppose olfactory excitation, just as suppressor band 19 and the other suppressor bands may have arisen from cortical needs for inhibition of optic, auditory, cutaneous and muscle sense stimulation, or the inhibitory centers in the formatio reticularis of the brain stem and cord may have been evolved for needs of inhibition from other cranial and spinal nerve excitation. To attain the required attention necessary for deliberation the cerebrum must be able to inhibit separately and collectively the effects of the various sensory stimulations.

This interpretation for the effects of lesions C and D has been considerably strengthened by the disclosure of this study that the required inhibition necessary for making correct conditioned foreleg responses from auditory and tactile stimulation was not impaired from lesions C or D, and the additional disclosure of a previous study (14) that a considerable ventral portion of the prefrontal cortex represented by lesions C or D received direct connections from the most important olfactory terminal of the brain, namely, the cephalic half of the piriform lobe. These connections were demonstrated (14) from recordings of electrical potentials following stimulations of the piriform lobe and from dissection of the uncinate fasciculus. Rose and Woolsey (15) have shown marked degeneration of cells in the median thalamic nucleus following extirpation of this region in the prefrontal cortex. It would appear that this nucleus is not the usual source of olfactory impulses for correct conditioned differential responses, since extirpation of the hippocampus or transection of the fornices (1) did not affect these responses, while ablation of the piriform lobes (16) abolished them.

Considerable data suggests that cortical inhibitory impulses are propagated extrapyramidally to lower motor centers. The writer presented evidence (17, 18) from spinal cord lesions, that stimulation of a small orbital prefrontal area in the rabbit no longer arrested respiration or slowed the pulse if the reticulo spinal tracts were eliminated. Dusser de Barenne and McCulloch (9), have reported that inhibition of motion, elicited from stimulation of 4s and other cerebral suppressor bands, is propagated extrapyramidally, independent of the caudate nucleus, corpus striatum, substantia nigra or cerebellum. McCulloch, Graf and Magoun (19), Verhaart and Kennard (10), Ward and McCulloch (20), Mettler (21, 22) and Papez (23) have either stimulated or extirpated suppressor band 4s or neighboring areas of the prefrontal region and obtained electrical potentials or Marchi degeneration in various relay areas in the brain stem.

On the other hand, it has never been disproved that the inhibition required for a correct negative condition reflex cannot be initiated by the negative conditioned stimulus suppressing the action of the motor cortex through timing or by some direct mode of action.

## SUMMARY

Bilateral, prefrontal lesions A or B (figs. 1 and 2) resulted in no permanent effects on the olfactory, auditory or tactile positive foreleg conditioned reflex. Occasional temporary effects occurred from slight injuries to the contralateral foreleg motor cortex.

Bilaterally, lesions C or D (figs. 3-5), while having no permanent effect on the positive olfactory conditioned reflex, abolished all correct foreleg conditioned differential responses from olfactory stimulation for over 5000 tests, which extended over periods of 6 months. Meanwhile, these correct responses could always be obtained from auditory or tactile stimulation.

The chief effect of lesions C or D on the olfactory differential tests was inability to withhold the foreleg response for the negative conditioned stimulus or lack of correct inhibition.

It is apparent that lesions C or D severed somewhere the circuit required for correct olfactory differential responses. Since the lesions extirpated Tower's (4) prefrontal inhibitory field for movement or suppressor band 8s of other authors, it seems likely that they removed the efferent center required for evoking correct negative conditioned reflexes from olfactory stimulation.

Consideration was given in the discussion to the possible function, origin and outlet of this prefrontal inhibitory area.

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# GASTRIC DISTENTION AS A FACTOR IN THE SATIATION OF THIRST IN ESOPHAGOSTOMIZED DOGS

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**I**N THE maintenance of constant water content of the body, mechanisms that initiate drinking behavior have received considerable attention. Those mechanisms which signal that the volume of water drunk is adequate for the needs of the organism require equal analysis. The present experiments elucidate one of the mechanisms operative in the satiation of thirst.

The dog, when in water deficit, will quickly drink an amount of water accurately gauged to make up that deficit (1, 2). Satiation occurs long before any appreciable volume of the ingested water has been absorbed from the gut (3). If a dog with an esophageal fistula is allowed to sham-drink, it will take an amount of water that is some 250 per cent of its real deficit before temporary satiation occurs. But, if a volume of water equal to the deficit is put into the stomach and the dog is immediately allowed to sham-drink, it takes an amount of water close to its real deficit. These observations were interpreted (4) as evidence for at least two factors which play a rôle in the satiation of thirst, a sub-fistulous factor and a factor operative above the esophageal fistula.

This investigation is a study of the quantitative relations between real and sham drinking, and of the rôle of the filling of the stomach as a factor in the satiation of thirst.

## GENERAL METHODS

The day preceding an experiment a dog with a surgically produced esophageal fistula was given only part of its water ration; sometimes sodium chloride was added to its food. On the experimental day the thirsty dog was placed in a stock, and after a rest period a pail of water was set before the animal. The water that poured out of the fistula was caught in a large beaker and measured. Most animals drank steadily, then stopped and left the water pail, thus giving a clear end-point to the drinking. At this point the water was removed. Since the satiation from sham drinking is short lived, tests of thirst intensity were run at hourly intervals in most of the experiments. The experimental period was usually alternated with control periods as illustrated in figure 2.

The esophagostomies were done in one stage under sodium pentobarbital anesthesia. Transthoracic vagotomies were also performed under the same anesthesia. All vagal strands were dissected free and removed from the 5-cm. section of the esoph-

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agus just above the diaphragm. The major nerve trunks were ligated with silver clips. It was believed that the resection of some 5 cm. of nerve plus the application of silver clips would make effective regeneration impossible. Completeness of the operations was verified at autopsy.

The stomach balloons used in these experiments were condoms fixed to a length of smooth soft-rubber tubing. They were passed into the stomach through the gastric stoma of the esophagostomy. For each experiment, the balloon remained in the stomach (empty or inflated) until the dog was ready to return to its cage. The balloon was filled and emptied, by gravity, from a calibrated reservoir of warm water that was raised above the dog's head just before each test, and lowered below its stomach level after each test. These motions of the reservoir were always carried

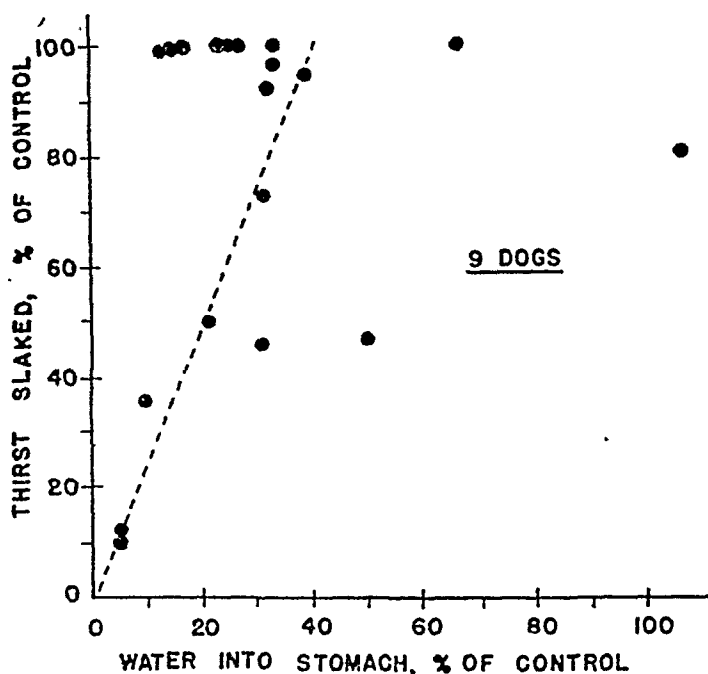


Fig. 1. THIRST slaked when water was put into stomach one hour previously.

out; the clamp on the connecting tube was only released when the balloon was to be filled.

In none of these experiments did the filling of the stomach balloon cause any signs of nausea.

## RESULTS

*Effect of Replacing Part of the Water Deficit.* After establishing a control level for sham drinking in 9 dogs, a volume of water, expressed as percentage of control sham drinks, was put into the stomach. After one hour, a time adequate for absorption, the dog was again allowed to drink. These experiments (fig. 1) indicate that absorption of roughly 40 per cent of the amount of water sham-drunk in the control tests is adequate to completely slake thirst. In other words, the dog sham-drinks some 250 per cent of its real deficit. We conclude that when the water swallowed is short-circuited (esophagostomy) and does not reach the stomach, the animal's ability to judge accurately the volume drunk is seriously impaired.

*Nature of the Distention Stimulus.* In normal drinking, the water entering the stomach could directly affect the mucosa, causing the release of a humoral agent which in turn acted upon central mechanisms to cause drinking to cease. On the other hand, the effect of the water might be just mechanical. By comparing the inhibitory effects on sham drinking of water free in the stomach versus water kept from contact with the mucosa by a balloon, one of the above hypotheses may be invalidated. If the main effect of the water is mechanical then the free water and the water in the balloon should give the same degree of inhibition. If, on the other hand, the mechanical rôle is a negligible one, there should be little effect on sham drinking when the water is contained within the thin-walled stomach balloon. Figure 2 represents a typical experiment on the effect of filling the stomach balloon with water just before alternate drinking tests. This experiment not only shows that balloon filling par-

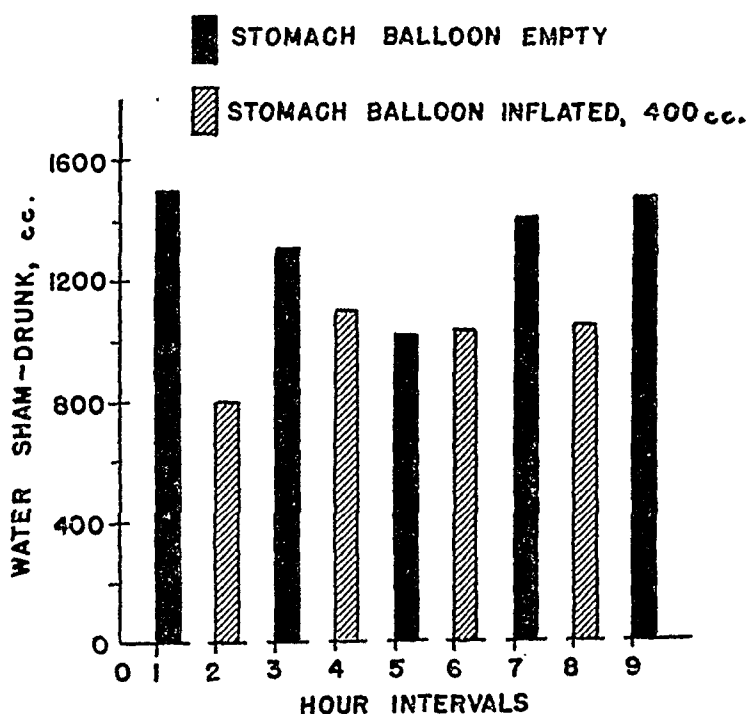


Fig. 2. INHIBITION of sham drinking by the inflation of a stomach balloon; typical experiment on dog 4.

tially inhibits sham drinking but it also gives some indications of the variability inherent in these experiments. Each of the solid dots in figures 3, 4 and 5 represents the averages obtained in one experiment like this.

The effect of water in the stomach was tested by first establishing a control level of sham drinking and then measuring the volume of water sham-drunk immediately after filling the stomach with some percentage of the control volume of water. We may first examine all the experiments done on some one dog (fig. 3); two experiments show the effect of water in the stomach and seven show the effect of stomach-balloon inflation. The hand-fitted line shows that if the stomach is inflated with a volume of water equivalent to the control sham drinks (100 per cent on abscissa) then the drinking will be only one third as great as the control drinks, a 65 per cent inhibition. It makes no difference if the water is in contact with the stomach or is contained in a stomach balloon.

Figure 4 is a plot of 20 'balloon' experiments on 6 dogs, and 11 'water' experiments on the same dogs. Although the lumping of data from 6 dogs makes the spread greater, in general the conclusions made with reference to the previous graph are applicable to the experiments in which the stomach balloon was inflated; increased stomach dilatation causes increased inhibition of sham drinking. The plot for the experiments in which the water was in contact with the mucosa shows no definite trend. This scatter may be explained by the fact that each of these points, open circles, represents just one test, whereas each of the solid points, 'balloon experiments,' represents the average of 3 to 6 tests.

The effect of the water in the stomach is mainly mechanical but, with these data, it is impossible completely to rule out some specific 'chemical' or contact effect that may play a contributory rôle. Though gastric distention is the most prominent feature of filling the stomach with water, factors such as pressure upon the abdominal

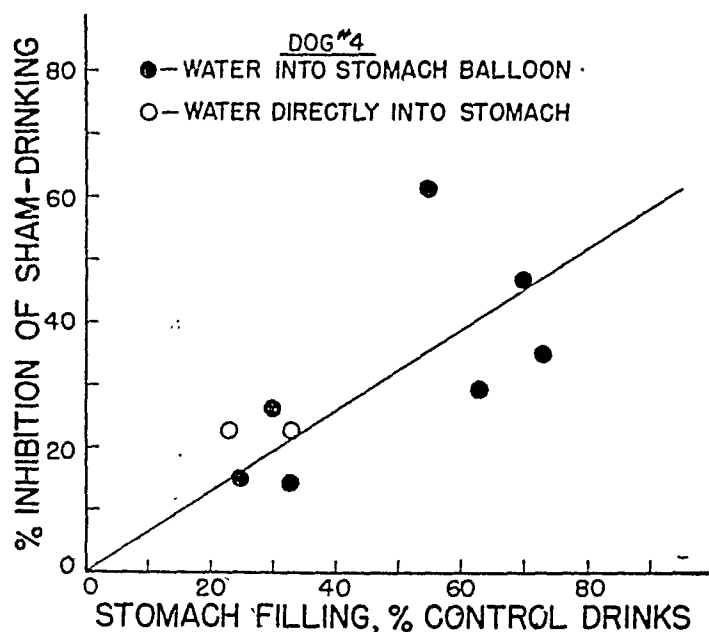


Fig. 3. INHIBITION of sham drinking by filling the stomach. One dog, 9 experiments.

wall and stretching of mesenteric attachments may also play a part. Such factors result from the weight of water and may be circumvented by inflating the balloon with air. In the one experiment where air inflation was used sham drinking was inhibited by about 50 per cent. The use of the word distention in this paper refers to the whole complex that may result when the stomach balloon is filled with water.

*Quantification of Inhibition of Sham Drinking by Distention.* To summarize the effect that filling a stomach balloon has upon the volume of water sham-drunk, the values for all the experiments performed have been plotted in figure 5. The hand-fitted line shows that with 100 per cent inflation of the balloon, drinking is inhibited by 60 per cent. This indicates that with the balloon empty, drinking was about 250 per cent of the real deficit, which checks closely with the results obtained in the experiments where part of the water deficit was replaced.

In other words, when 100 per cent of the control volume is put into the balloon the inhibition is sufficient to bring the sham drinks down to about one third of their

control size, or down to the range of the real deficit. It seems that in drinking the distention of the stomach plays a rôle in the estimation of the volume of water to be ingested.

*Individual Sensitivity to Gastric Distention.* Are all dogs equally sensitive to stomach distention? In part A of table 1 are summarized the mean results for all experiments performed on each of 10 dogs. From these data, the inhibition in sham drinking that would result from filling the stomach balloon with a volume of water equal to the control sham drinks has been calculated. Implicit in such a calculation is the assumption that percentage inhibition of drinking and percentage balloon inflation are linearly related. This assumption receives some support from figures 4 and

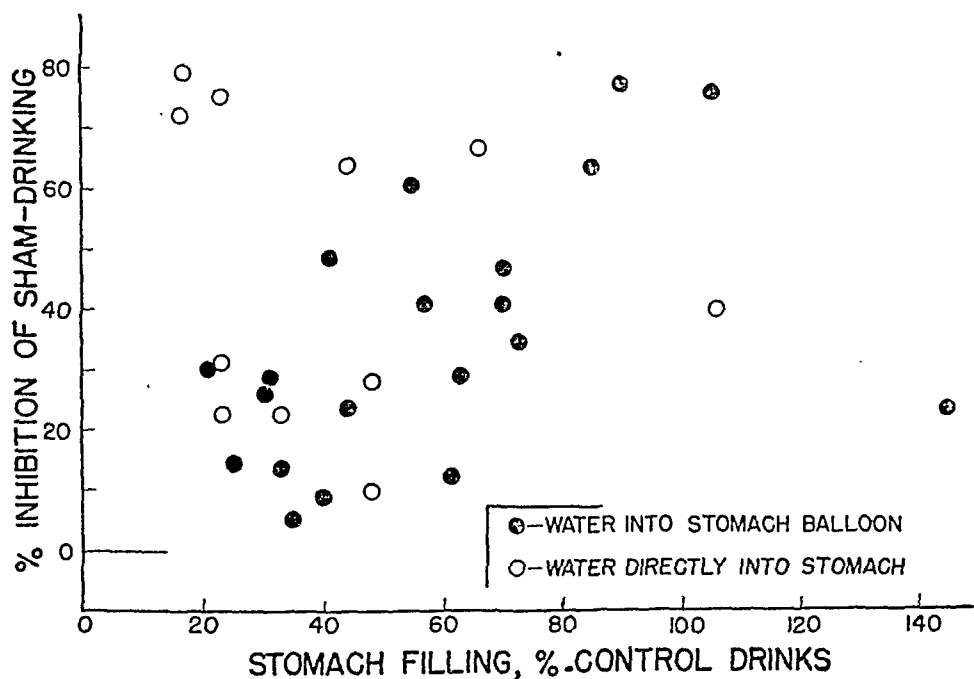


Fig. 4. EFFECT upon sham drinking, in 6 dogs, of water put in stomach balloon and of water put directly in the stomach.

5. These calculated values for inhibition give a basis for comparison among the 10 dogs.

The animals exhibit individual variations to the distention stimulus; inhibition ranged from 26 to 78 per cent with a mean value of 57 per cent. So, though all dogs tested make use of distention cues, they seem to vary in their dependence upon these cues.

*Effect of Gastric Distention upon Ad Libitum Sham Drinking.* During the course of the above experiments it was observed that when the stomach balloon was inflated the dogs seemed quite content after taking their sham drinks, often curling up and going to sleep. Whereas, when the balloon was empty there was restlessness and gazing toward the sink from which the water pail came.

In addition to these qualitative observations a few experiments were designed to show how the dog sham-drinks when the water is available for a long period, and what effect the filling of the stomach balloon has under these circumstances. Results of one such experiment are plotted (fig. 6). The 3 hours during which water was

available are spaced by 2 hours when the pail had been removed. Despite the huge draft of over 4 liters, the dog took three additional drinks during the next 7 minutes, the stomach balloon being empty. In the next drinking hour the inhibition in drinking caused by inflation of the balloon not only reduced the first drink to a bit over 2 liters, but only one additional drink was taken during the next 25 minutes. After the balloon had been filled for about one half hour, the dog sham-drank much as it did when the balloon was empty. The third drinking hour starts out with the balloon empty and is a somewhat more typical drinking pattern than the one of the first hour. When the balloon is filled in the second half of this hour, only two drinks are taken.

That distention of the stomach plays a rôle in the satiation of thirst is again evidenced by the decrease in the frequency of drinking in such experiments as this.

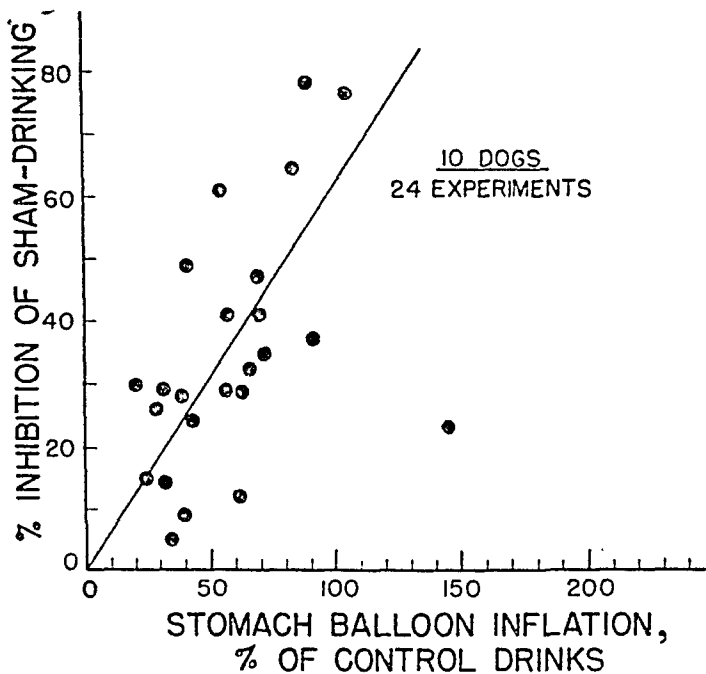


Fig. 5. INHIBITION of sham drinking by inflation of stomach balloon.

*Pathway of the Distention Cue.* Since distention of the stomach is one of the cues for thirst satiation in the dog, a question arises: what is the path of the afferents for such a cue? The distention stimulus may be transmitted to the central nervous system by humoral or nervous means, or by both.

What suggestions may be obtained from previous investigations? Humoral transmission of gastric distention stimuli have been shown (5) to participate in the control of gastric secretion in the dog. Sensory endings in gastric muscle, such as might be capable of mediating the sensation of distention, have been described (6). In the cat 'distention' afferents from the stomach have been shown to be in both vagi and in the sympathetic chain when pupillo-dilation was the observed response (7). Attention was focused mainly on the vagus, for it has been assigned, in classical physiology, the rôle of mediating impulses from the stomach that are responsible for satiety of hunger (8).

Supradiaphragmatic vagotomy was performed on 5 dogs and the effect, on

drinking, of balloon distention of their stomachs was tested. Part *B* of table 1 summarizes these experiments.

In general, inflation of the balloon to the control-drink volume produces approximately a 57 per cent inhibition in drinking prevagotomy, but only a 15 per cent inhibition postvagotomy. Three of the 5 dogs take drinks, with the balloon inflated, as large or larger than their control drinks. The balloon distention no longer has any effect on drinking in these dogs. *Dog 6* shows a 36 per cent inhibition postvagotomy but there are no preoperative experiments on this animal for comparison. However, this value is below the general trend of sensitivity for the dogs that were

TABLE 1. EFFECT OF INFLATING A STOMACH BALLOON UPON SHAM DRINKING

DOG NO.	NO. OF EXPERIMENTS	BALLOON EMPTY: MEAN SHAM-DRUNK	MEAN BALLOON INFLATION VOLUME	BALLOON INFLATED: MEAN SHAM-DRUNK	PER CENT INHIBITION IN SHAM DRINKING IF BALLOON WERE INFLATED TO CON- TROL DRINK VOL.
		cc.	cc.	cc.	
<i>A. Prevagotomy</i>					
1	1	1025	950	650	40
2	1	1347	500	963	78
4	7	1034	422	764	63
5	1	668	450	453	47
7	3	1617	630	1217	64
8	2	443	466	112	71
11	1	1028	583	732	51
12	1	430	366	155	75
15	3	1790	850	1568	26
16	4	1691	800	1240	57
					Mean = 57
<i>B. Postvagotomy</i>					
6	1	2356	980	2010	36
8	2	392	467	395	-1
12	1	655	300	680	-9
15	2	780	790	802	-3
16	2	1865	830	1419	53
					Mean = 15

tested prevagotomy. There are both pre- and postvagotomy experiments on *dog 16*, and in this dog vagotomy does not seem to have produced any change in sensitivity to the balloon. At autopsy this vagotomy was grossly complete and one must suppose that this dog made use of distention cues other than those that might vary impulses that ascend the vagus.

The individual experiments for those dogs where there are both pre- and postvagotomy experiments have been plotted in figure 7. The data are keyed, as indicated in table 1, so that the experiments for any one dog can be differentiated from the others. All the postvagotomy experiments show less inhibition of drinking than the preoperative experiments in the same dog except one experiment for *dog 16*. This one value is even out of the range for the preoperative data.

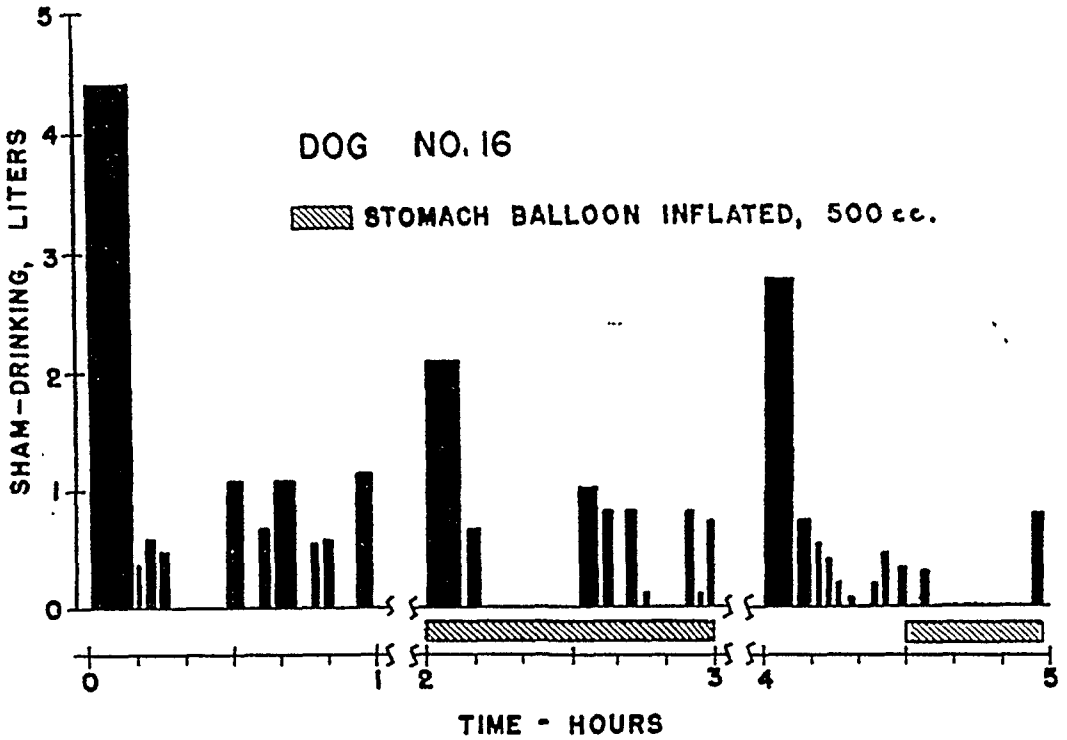


Fig. 6. FREQUENCY of sham drinking as affected by stomach balloon inflation.

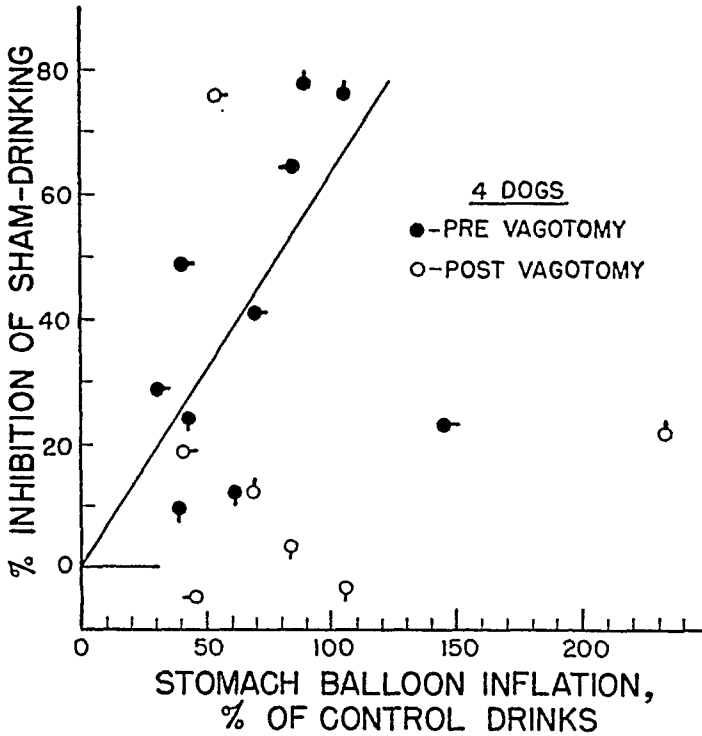


Fig. 7. VAGOTOMY, as it affects sensitivity to stomach balloon inflation. The dogs may be differentiated by the keys on the points. See table 1.

Vagotomy in the esophagostomized dog markedly reduces the inhibition of sham drinking caused by stomach distention. Some dogs may not rely completely upon vagal pathways for the transmission of the distention stimulus.

## DISCUSSION

The satiation of thirst that immediately follows drinking is apparently brought about by the summation of afferent impulses that result from the drinking itself. However, this is a temporary satiation, for if no water actually enters the internal environment (esophagostomy) the dog will, within a few minutes, again exhibit drinking behavior. The factors that enter into this initial temporary satiation are probably numerous, and include the afferent activities initiated by the filling of the stomach. The 'distention' cue is only a part of the complex that enables the dog to judge the volume of water drunk, for even when deprived of this cue (esophagostomy) the dog does stop drinking. Without the distention cue, the other components of temporary satiation must overwork before drinking is inhibited; this results in over-drinking.

The author postulates that the 'distention' cue, along with other as yet unidentified cues, acts, in the fashion of negative feed-back, to inhibit drinking. When the feed-back is enough to counteract the factors which have to do with initiating drinking behavior a state of temporary satiation exists. Subsequent to the absorption of water the factors initiating drinking behavior are removed and then a state of relatively permanent satiation exists.

## SUMMARY

A series of experiments upon sham drinking in esophagostomized dogs when in water deficits revealed the following: 1) The dog sham-drinks some 250 per cent of its real water deficit before temporary satiation occurs. This indicates that when the water drunk does not reach the stomach the animal's ability to measure its intake is seriously impaired. 2) Distention of the stomach reduces the volume of sham drinking and the frequency of sham drinking so that sham drinking takes on the characteristics of normal drinking. 3) Distention of the stomach may be accomplished by a water-filled stomach balloon with results similar to those in which water was in contact with the mucosa. Mechanical factors account for the major effect of stomach filling as far as inhibition of drinking is concerned. 4) All dogs tested make use of distention cues but they vary in their dependence upon these cues. 5) The main afferent pathway for impulses initiated by gastric distention and concerned with the regulation of drinking probably ascends the vagi; for, following vagotomy, distention of the stomach has almost no effect upon sham drinking. 6) Satiation of thirst includes an immediate temporary satiation and a subsequent permanent satiation which follows the intestinal absorption of water.

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# STUDIES IN CALCIUM URINARY EXCRETION WITH THE AID OF RADIOCALCIUM<sup>1</sup>

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SEVERAL investigators have used radiocalcium as a tracer in the study of calcified tissues. Campbell and Greenberg (1) studied the retention and excretion of  $\text{Ca}^{45}$  given by stomach tube to a rat. As has been shown by Greenberg (2), vitamin D promotes the absorption of calcium from the digestive tract. Experiments performed by Pecher (3) indicate a selective concentration of radiocalcium in the skeleton giving a method to irradiate the skeleton selectively for therapeutic purposes. Recently Armstrong and Barnum (4) studied the metabolism of calcified tissues by the simultaneous use of radioisotopes of calcium and phosphorus.

We have used radiocalcium to test the mechanism of excretion of calcium by the kidney. Our previous observations (5) with radiophosphorus in the study of excretion of phosphorus indicate that exogenous phosphate is handled differently from endogenous phosphate by the kidney. During the first hour after an intravenous injection of labelled disodium phosphate solution into dogs, the urine receives a higher proportion of exogenous over endogenous phosphate than would be expected judging from the ratio of the two types of phosphate found in the plasma. After  $1\frac{1}{2}$  hours, the plasma and urine ratios approach equality. From our observations we may conclude that the physico-chemical state of an important fraction of the so-called inorganic phosphate in the plasma is different from that of the injected phosphate. Only a small fraction of the acid-soluble phosphate is in a pure inorganic, rapidly exchangeable form.

Friedlander and Wilde (6) recently stressed the importance of this observation as it bears upon their method of calculating plasma to tissue exchange rate by substituting the terminal urinary isotope ratio in place of the integrated plasma value. The calculation of exchange rate requires a precise evaluation of the ratio of the tracer and non-tracer isotope entering a tissue from the plasma. The urine ratio enables one to check on this.

## EXPERIMENTAL DETAILS

Radioactive calcium was produced by the pile at Clinton Laboratories, Oak Ridge, Tennessee. The radioactive material had a high specific activity (0.015 mc/mg.). The radiocalcium was in the form of  $\text{CaCl}_2$  dissolved in water.

Dogs were anesthetized by chloralose and the ureters were catheterized. Blood samples were taken from the femoral artery. The animals received intravenously, labelled calcium chloride solutions containing 0.373 to 90 mg. calcium and about 0.310 counts per minute of  $\text{Ca}^{45}$ . Before and immediately after administration of the calcium solution, blood samples were taken and small samples of urine collected.

Radioactivity measurements were made with a thin mica window Geiger-Müller tube. All measurements were corrected for the resolving time losses and for back-

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<sup>1</sup> The  $\text{Ca}^{45}$  was furnished by the U. S. Atomic Energy Commission.

ground. An effort was made to keep the corrections for resolving times as low as possible. All samples were dry ashed at 500°C. and were placed as dry samples with

TABLE I. SERUM DATA

TIME <sup>1</sup>		TOTAL SERUM Ca	Ca <sup>45</sup> <sup>2</sup>	EXOGENOUS OR INJECTED SERUM Ca		SPECIFIC ACTIVITY
hr.	min.	mg/cc.		μg/cc. <sup>3</sup>	% of total	
0	0	0.1130				
0	6	0.1145	0.073	0.272	0.24	0.637
0	11	0.1270	0.060	0.227	0.18	0.479
0	32	0.1118	0.039	0.145	0.13	0.348
0	52	0.1270	0.039	0.148	0.12	0.313
1	30	0.1290	0.041	0.154	0.12	0.320
2	0	0.1140	0.027	0.100	0.09	0.236
3	19	0.1190	0.026	0.096	0.08	0.218
4	17	0.1110	0.021	0.081	0.07	0.196
5	2	0.1160	0.017	0.063	0.05	0.147
5	42	0.1007	0.018	0.067	0.06	0.179
6	25	0.1100	0.014	0.053	0.05	0.129

<sup>1</sup> Time after injection at which blood sample was drawn.

<sup>2</sup> In 1 cc. of serum, expressed as percentage of the total dose of activity.

<sup>3</sup> Calculated by multiplying the proportion of Ca in μg to counts in the injected material by the number of counts in 1 cc. of serum or urine.

TABLE IA. URINE DATA

TIME <sup>1</sup>		URINE COLLECTED	TOTAL URINE Ca	Ca <sup>45</sup> <sup>2</sup>	EXOGENOUS OR INJECTED URINE Ca		SPECIFIC ACTIVITY
hr.	min.	cc.	mg/cc.		μg/cc. <sup>3</sup>	% of total	
0	2	29.0	0.0878				
0	3.5	6.7	0.0850	0.072	0.270	0.31	0.852
0	7.5	9.4	0.0780	0.051	0.191	0.24	0.658
0	15	8.0	0.0910	0.043	0.161	0.17	0.475
0	30	7.0	0.0850	0.031	0.117	0.13	0.369
0	44	19.5	0.0691	0.021	0.079	0.11	0.309
0	57	23.0	0.0642	0.018	0.070	0.10	0.292
1	13	13.5	0.0624	0.020	0.074	0.11	0.320
1	34	10.0	0.0585	0.015	0.057	0.09	0.261
1	56	6.5	0.0438	0.014	0.054	0.12	0.333
2	19	18.5	0.0682	0.011	0.044	0.06	0.174
2	56	18.0	0.0244	0.008	0.031	0.12	0.340
3	47	6.5	0.0273	0.008	0.032	0.11	0.319
4	39	6.0	0.0244	0.005	0.020	0.08	0.217
5	46	5.0	0.0370	0.005	0.018	0.04	0.135

<sup>1</sup> Time after injection at which urine sample was collected.

<sup>2</sup> In 1 cc. of urine, expressed as percentage of total dose of activity.

<sup>3</sup> See footnote 3, table 1.

identical geometry under the counter. Standards prepared in the same manner were also measured. The reproducibility of such procedure is indicated by duplicate radioactive measurements of serum which agreed within 3.4 per cent.

Blood was allowed to clot and the serum collected. Calcium was precipitated as oxalate and determined chemically according to the standard analytical procedure.

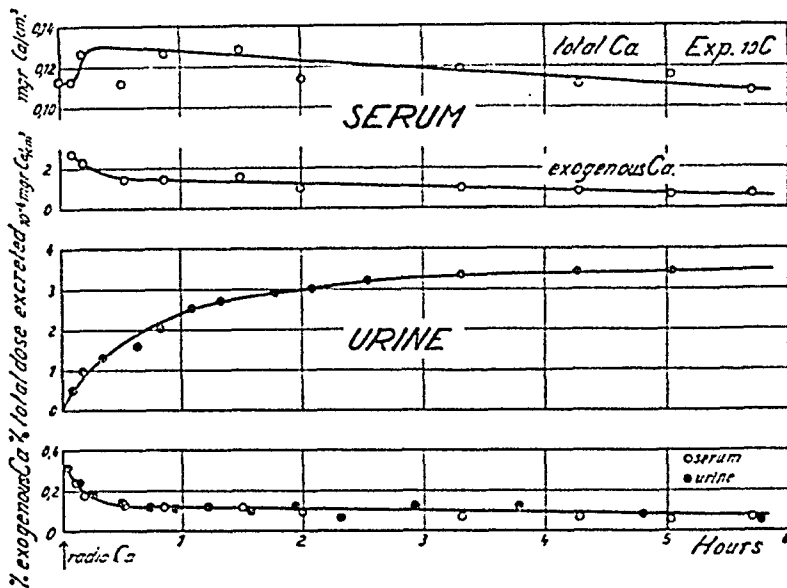


Fig. 1. VARIATION of total Ca in serum. Rate of disappearance of the injected calcium in serum. Cumulative excretion of injected calcium by urine. Variation of exogenous calcium as a percentage of total calcium present in the plasma and in the urine. Radiocalcium injected at time zero.

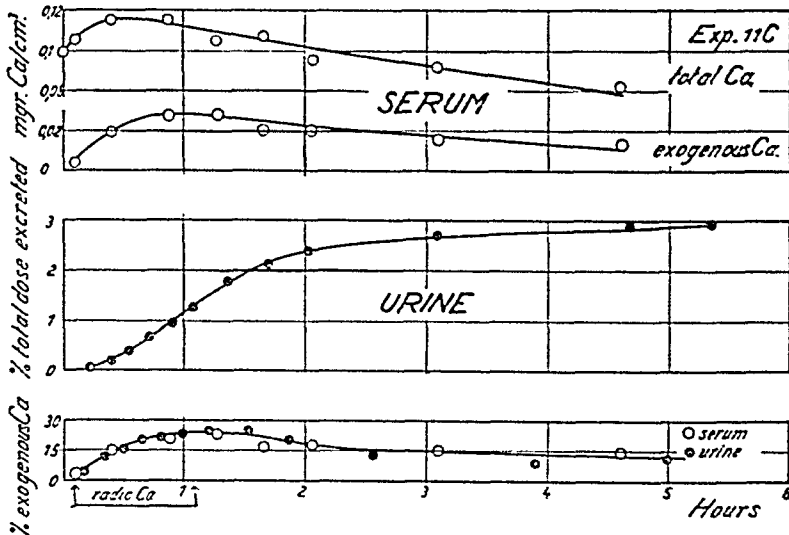


Fig. 2. VARIATION of total Ca in serum. Rate of disappearance of the injected calcium in serum. Cumulative excretion of injected calcium by urine. Variation of exogenous calcium as a percentage of total calcium present in the plasma and in the urine. Radiocalcium injected drop by drop during one hour.

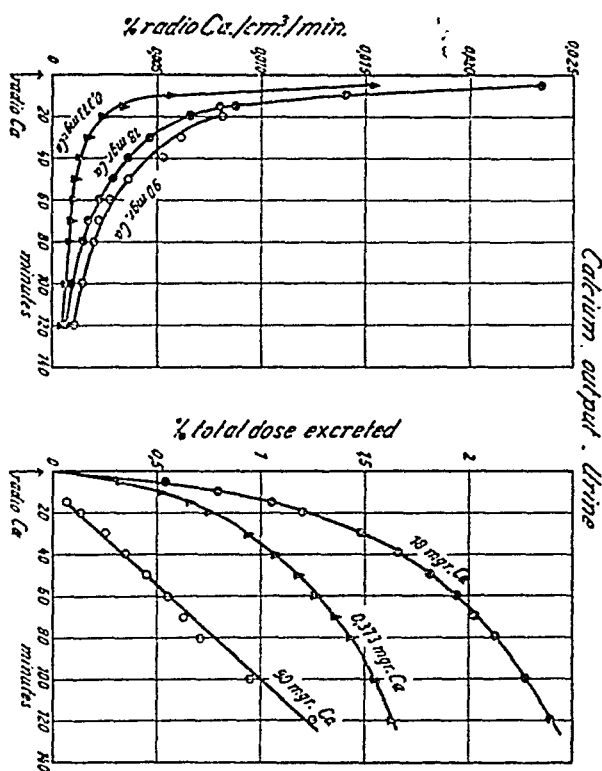
#### RESULTS AND DISCUSSION

The data obtained in one typical experiment are shown in table 1. The dog weighed 5.5 kg. and received 0.373 mg. labelled calcium chloride. Figure 1 shows graphically the variation of total and exogenous calcium in serum and the cumula-

tive excretion of tagged calcium in urine during about 6 hours following injection. The variation of exogenous calcium expressed as percentage of total calcium present in the plasma and in the urine is also shown in figure 1.

The exogenous calcium appears to be an equal proportion of calcium in serum and in urine even for the first samples of urine collected after injection. Similar results are obtained by injecting smaller or larger quantities of calcium, even when the calcium solution is injected drop by drop over longer time as shown in figure 2. Figure 3 shows that the rate of calcium excretion increased with the injected amount. Nevertheless the absolute quantities of calcium excreted are lowered when too large doses of calcium uncompensated by potassium are injected. This accompanies a fall in urine output.

Fig. 3. INFLUENCE of total amount of calcium injected on rate of urinary excretion of radio-calcium and on cumulative elimination in the urine. Each curve represents the mean data obtained with different animals.



The excretion of calcium in the urine is different from the excretion of phosphorus. Exogenous calcium is not handled differently from endogenous calcium by the kidney. The injected calcium, in pure inorganic form, is excreted by the kidney at the same rate as endogenous calcium of the plasma. In view of the results obtained with radiocalcium we may conclude that the differences observed between the excretion of calcium and phosphorus by the kidney cannot be attributed to the radioactivity of the tracer. The possibility of an isotopic separation is also no more likely for phosphate than for calcium.

#### SUMMARY

Radioactive  $\text{Ca}^{45}$ , with a half life of 180 days, was used in tracer studies concerning urinary excretion of calcium. Radioactive measurements show that exogenous calcium, unlike phosphate, is excreted into the urine in proportion to its abun-

dance in plasma as a percentage of total Ca in the latter system. The percentage of exogenous  $\text{PO}_4$  found in urine exceeds that for plasma.

I am indebted to Professors L. Brull and G. Guében who sponsored this work. My thanks are due to Prof. L. Brull for making the animal preparations. I wish to express my appreciation to Miss Vossins, Miss Grisard and Mr. Bezzan for technical assistance.

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# KETOGENIC ACTION OF NIACIN IN THE NORMAL FASTED RAT<sup>1</sup>

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IOWA CITY, IOWA

EARLIER it was shown that a ketonuria resulted if excessive amounts of niacin were fed to severely diabetic rats (1). This observation was somewhat surprising since there was no known explanation for the ketogenic action of this compound. The present work is concerned with the study of the effect of niacin on the production of ketone bodies in the normal rat. Since it is difficult to produce a ketosis in the rat, the method of fasting as employed by Deuel and Hallman (2) has been used.

## EXPERIMENTAL

Female rats of the Long-Evans strain, 6 to 8 months of age, were used. Niacin was dissolved in physiological saline and made up so that a given amount was contained in 5 cc. of the solution. One intraperitoneal injection was given daily. Control rats received 5 cc. of saline.

The rats were kept in metabolism cages. Urine was measured daily and tested for acetone by the sodium nitroprusside method. In all experiments total urinary acetone bodies were determined on the 3rd and 5th days of fasting by the method of Van Slyke. In certain instances, these determinations were also made just preceding the fast and after 7 days of fasting.

The ketogenic action of niacin was studied in both fasting and nonfasting animals. The following experiments were conducted: 1) Non-fasting rats were injected with 35 mg. of niacin per day for 5 days while receiving a basal synthetic diet<sup>2</sup> which contained 18 per cent protein (3). This niacin solution had a *pH* of 3.6. 2) Animals were fed a diet containing an excessive amount of niacin (1 mg./gm. of diet) previous to a 5-day fast. During the fasting period the rats were injected with 35 mg. of niacin per day. 3) The effect of other levels of niacin was also investigated. 4) In addition to the experiments with niacin, the ketogenic activity of two acids and two vasodilators was studied.

The non-fasting rats which received excessive amounts of niacin for 5 days usually showed no acetonuria.<sup>3</sup> This was not surprising, however, because the rat

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<sup>2</sup> The vitamins and prostigmin were supplied through the kindness of Hoffmann-La Roche Inc.; the mecholyl chloride by Merck & Co., Inc.

<sup>3</sup> The average acetone body excretion for 8 rats was 0.3 mg. during the last day of injection.

is apparently very efficient in metabolizing fats as evidenced by the difficulty of producing ketosis in this animal (4).

In contrast to the non-fasted rats, the majority of animals which were fasted and given excessive amounts of niacin showed a marked acetonuria. This was evident in the series of rats which was fed the diet containing excessive amounts of niacin and then fasted for 5 days while receiving niacin injections. Only a mild acetonuria was observed previous to the fast (table 1). On the 5th day of fasting the urine from most of the rats gave a 4 plus test for acetone, while that of the saline controls was usually from 0 to 2 plus.

The daily dose of 35 mg. of niacin was an arbitrary value based on the average amount ingested by diabetic rats which had showed a ketonuria. The effect of daily injections of 5, 20 or 50 mg. of niacin was also investigated. Urinary acetone values for these rats as well as for some prefast and fasted controls are shown in figure 1. The treated animals varied in their response to the drug, some failing to show a ketonuria, but the 35 and 50 mg. levels seemed to be the most ketogenic. One value

TABLE 1. URINARY ACETONE BODIES, MG/24 HOURS (AVERAGES)

TREATMENT	DIET	NO. RATS	WT. CHANGES DURING EXPR. PERIOD	AV. FOOD INTAKE/ DAY FOR 5 DAYS	ACETONE		
					Prefast	3rd Day of fast	5th Day of fast
Saline	Basal prior to 5-day fast	7	gm. — 29	gm. 11	0.37	1.24	1.96
Saline prior to fast; 35 mg. niacin during fast	Hi niacin prior to 5-day fast	7	— 29	12	2.50	11.46	15.31

for an animal which received 5 mg. of niacin daily is not shown on figure 1 as it reached 89.24 mg. on the 5th day. The diet fed the animals previous to fasting, which was either Rockland rat ration or the basal synthetic diet, did not affect the response to niacin.

In an effort to determine how long the ketonuria would persist after niacin injections were discontinued, a group of rats was injected with niacin during the 5-day fasting period and then fasted 2 additional days, at which time they were injected with saline. The excretion of acetone bodies returned to normal or near normal levels at the end of the 7-day fast. This is in agreement with observations on diabetic rats. When the amount of niacin was reduced to the requirement level, the acetonuria usually disappeared within 2 days (1).

The question arises as to whether the excretion products which were present in the urine were acetone bodies or some compound which might give the test for acetone; and whether the metabolism of niacin per se might yield acetone bodies. As to the first point, a verification test (5) showed indirectly that the excretion products, calculated as acetone, made up about 98 per cent of the precipitate. Most of the acetone bodies were in the form of beta-hydroxybutyric acid. As to the metabolism niacin, it is very unlikely that the excretion products could give the test

for acetone. Nevertheless, compounds closely related to niacin which were available in the laboratory, some of which would be present in the urine only under extreme

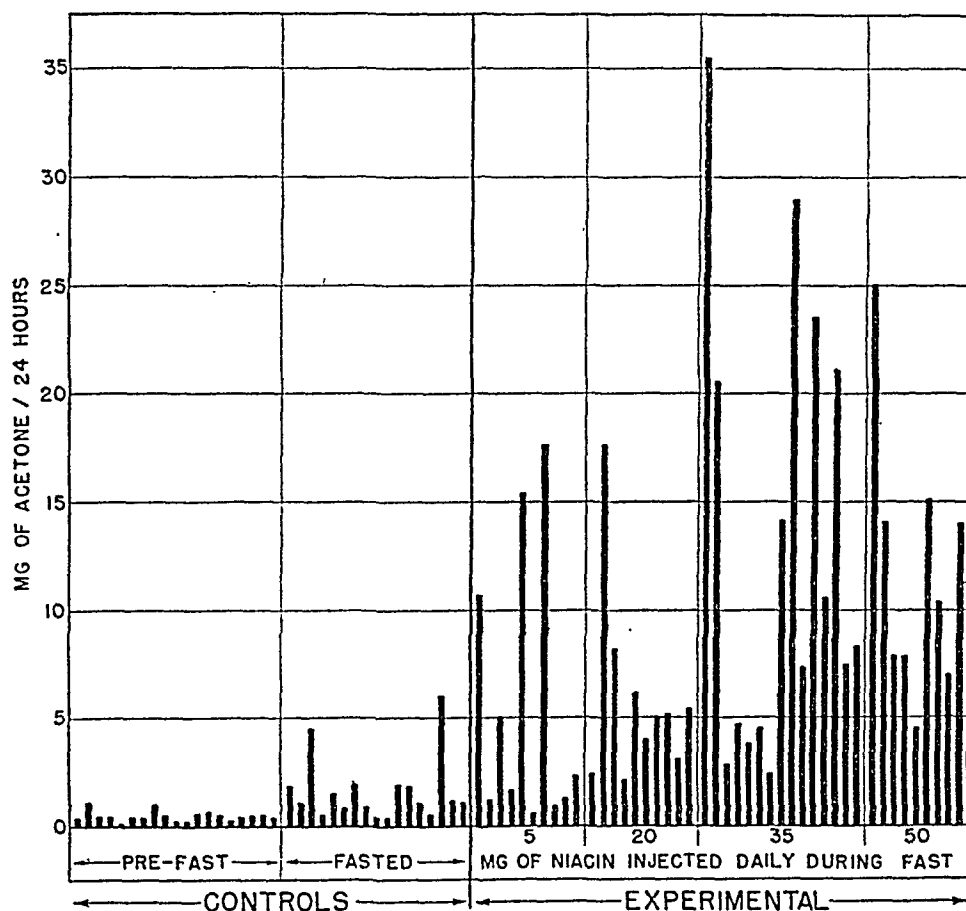


Fig. 1. EFFECT OF NIACIN on the excretion of urinary acetone bodies (individual values for last 24 hours of 5-day fast).

TABLE 2. EFFECT OF OTHER ACIDS AND VASODILATORS ON URINARY ACETONE BODIES

TREATMENT	pH OF SOLUTION	NO. RATS	TERMINAL WEIGHT gm.	URINARY ACETONE BODIES MG/24 HOURS	
				3rd day of fast	5th day of fast
Saline control	6.5	15	207	1.09	1.73
Niacin, 35 mg/day	3.6	15	202	8.67	13.03
Prostigmin bromide, 3 mg/day	7.2	8	219	0.65	0.53
Mecholyl chloride, 10 mg/day	6.5	8	200	0.83	1.46
Hydrochloric acid	3.4	8	205	0.37	1.07
Propionic acid	3.1	8	224	0.65	1.15

pathological conditions, were studied. Methyl nicotinamide, trigonellin, nicotinic acid, nicotinamide, coenzyme I, sodium nicotinate and acetyl pyridine hydrochloride were tested, and none of these substances produced the characteristic complex acetone precipitate with the Van Slyke method.

The acid and vasodilatory properties of niacin were of interest in relation to the ketogenic action of this compound. Accordingly, two acids and two vasodilators were given normal fasted rats. Unfortunately, an aromatic acid was not available which had the same ionization constant as niacin and yet was sufficiently soluble without being toxic. Solutions of hydrochloric acid and propionic acid with approximately the same  $pH$  as the niacin solution were used, and although propionic acid is probably antiketogenic (6) neither acid caused an increase in the production of acetone bodies (table 2). Likewise it was virtually impossible to find compounds which induce a vasodilation similar to that produced by niacin. Two compounds, mecholyl chloride and prostigmin bromide, which are vasodilators in human beings, were given to fasted rats but caused no increase in the elimination of ketone bodies (table 2). The prostigmin was dissolved in 5 cc. of distilled water and given by stomach tube.

It is not possible to state at the present time whether the ketonuria induced by niacin results from an over-production or an under-utilization of acetone bodies. In the event that differences in lipids of the liver might help to answer this question, some of the livers have been studied by cytochemical methods. However, no marked differences have been noted in the amount or the distribution of the lipids in the ketotic and non-ketotic rats.

#### SUMMARY

The daily intraperitoneal injection of excessive amounts of niacin into normal fasted rats of the Long-Evans strain, for periods up to 5 days, produced a marked ketonuria. The ketogenic action of this compound is probably not associated with its acid or vasodilatory properties.

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# THE AMERICAN PHYSIOLOGICAL SOCIETY PROCEEDINGS

## FALL MEETING

*Augusta, Georgia, September 14–17, 1949*

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### PHYSIOLOGY ON HORSEBACK<sup>1</sup>

WALLACE O. FENN

*President, The American Physiological Society, 1946–1948*

THE BEST AFTER-DINNER SPEECH I ever heard before this Society was one given by Professor Carlson, then President of the Society, in Cleveland in 1925. I am not sure just what he said but I am sure that it was highly entertaining to the Society. One thing I do remember. He told us about his dream. He dreamed that he went to heaven and was strolling down the golden streets and happened to run into the late and much lamented Dr. Cannon. Unfortunately, Dr. Cannon was not as happy as one should be in that environment and Dr. Carlson inquired very solicitously about the reason for his troubles. Dr. Cannon heaved a sigh and explained that everything was really very lovely—indeed it was too lovely and that was just the trouble. Nothing exciting and unexpected ever happened and there was no emergency and therefore no need for his adrenal glands.

Those were days of peace and comparative quiet. In these troubled times some of us may look forward a little wistfully to a no-emergency time in the Happy Research Grounds in the good company of Dr. Cannon and many others whose names are honored by members of this Society. I like sometimes to think that the life-hereafter will offer plenty of interest and excitement even though it may be free of emergency in the strict sense;

<sup>1</sup> Past President's address, read at the dinner of the American Physiological Society, at the First Fall Meeting, Minneapolis, Minnesota, September 16, 1948. Published at the request of the Council.

I hope it will offer us a complete access to the knowledge of life processes for which we now strive so strenuously and devotedly, a backstage view of physiological processes. At the end of my course on physiology I sometimes use a lecture or lectures on muscular exercise as a convenient means of summarizing the material previously presented. For in times of stress all the physiological mechanisms of the body are more or less involved in one way or another. To dramatize this subject I once tried the rather whimsical trick of presenting it as a Physiologists' Baseball Game in the Elysian fields. All the great physiologists of the past were present in the grandstands and all the great military heroes of the past such as Ajax, and Odysseus, Achilles and Hector were participating in the game. Each physiologist was equipped with a special pair of glasses designed to filter out and reveal to the wearer the particular bodily processes or the particular chemical substance in which he was interested. If you had the pH glasses and wanted to change them for membrane potential or bodily fatigue or Ca or ATP glasses you merely applied to the angel in charge and signed a receipt. Thus Harvey had the circulatory goggles, Lavoisier, the respiratory goggles, and perhaps Pavlov was catching up on the CNS with brain-wave glasses. Helmholtz with the visual goggles is particularly excited over the exact mechanism of color vision which he now observes for the first time and he watches with keen interest when the ball is pitched and observes the many mechanisms

by which the tiny image of the ball flitting rapidly over a few cones in the fovea initiates with breathtaking perfection the beautifully coordinated movements of the whole body which result in a home run by the wily Odysseus right over the backfield fence into hell. DuBois Reymond is there too with Galvani, both looking through nerve-muscle goggles. When Odysseus passes the home plate in a state of some fatigue, DuBois Reymond leaps up in great excitement and slaps Galvani on the back and says, "Look how the potassium and acetylcholine are piling up in his myoneural junctions. Isn't that beautiful!" Thus all the secrets which they strove so hard to discern throughout their working lives are now revealed to them clearly and painlessly in all their beautiful details. No wonder they are as excited and enthusiastic as any real-life baseball fan over the World Series.

Enough, however, of this Alice in Wonderland stuff! It wasn't much of a lecture and I mention it only because it illustrates the sort of a paradise which a physiologist would like to experience on earth—a paradise which he is in danger of losing in the stress of present conditions. What we want is to fill our hearts with wonder over the beauty of life processes, our minds with intellectual curiosity over the mechanisms employed, and our waking hours with earnest efforts to discover and understand and where possible apply these mechanisms.

What I wish to say this evening is that there are influences abroad which may destroy this type of paradise if we are not careful and that much of the danger lies in the fact that physiology has now taken to horseback like its elder sisters, physics and chemistry.

The first World War came and went and left physiology as an infant science in this country. Some physiologists worked during the war on shock or aviation medicine but on the whole this was a minor effort. The status of physiologists was not materially changed by that conflict. It was still a science for the Ivory Tower and few young men were going into physiology for a career.

I need not remind you that the last war has changed all that. One sign of the times is the founding of the *Journal of Applied Physiology*. Industry beckons with a strong arm. The persua-

sive call of the Almighty Dollar has penetrated to the once quiet recesses of the laboratory. The portentous international situation has cast its spell over us and the urgency of National Defense problems has diverted our former interests into new channels of military significance which now claim varying proportions of our time. Suddenly we have found ourselves projected from our Ivory Towers into the Marts of Men. The 'long-haired boys' have left their laboratories and are out with the Men on Horseback in the military camps, on the sea and in the air, and in all the unfriendly environments from the pole to the equator. By way of personal example I hope you will pardon me if I mention, not without a touch of pride, that ten days ago I went snorkeling in a submarine and dove 25 fathoms deep in the waters of Long Island Sound. What business had a pre-war civilian physiologist in such a place!

The war of course had a great effect on all sciences. The atom bomb and radar have sold scientific research to the public in a very convincing manner and for large sums of money. I really believe, however, that it changed the status of physiologists (or biologists in general), and perhaps of psychologists, more than that of other scientists. The professional stock of both these groups rose to unprecedented levels and their services were and still are in demand and apparently highly appreciated by military men. As a sign of the importance of physiology to the military may I mention that in the new Committee of Medical Sciences of the Research and Development Board, the three civilian members appointed were assigned, one to surgery, one to medicine and one to physiology. This is a clear indication of the number of military problems which came within our range of competence.

All this is very flattering and advantageous to physiologists. We now have something to sell; our services are in demand. Long live the physiologist says an appreciative world. "On what?" says the physiologist. Now at last there is a good prospect that there will be at least a living for all the physiologists we can train. Without an adequate market for its students no science can really thrive because it will not attract the best brains; competition will not be keen enough for the best selection and there will be too few drones to sup-

port the queens. In the future every student can expect a job. The danger is that in gaining a living he may have to prostitute his intellect for an applied problem of commercial or military importance which is devoid of fundamental significance.

The need for basic research is perhaps illustrated in an amusing way by a story which I am told has been going the rounds of the Navy recently. A man called up a veterinarian about his sick cat, and described its symptoms. The veterinarian understood calf for cat and prescribed a pint of castor oil which was duly administered (more or less). Some days later the veterinarian met his client and inquired about the welfare of the patient. The man threw up his hands in despair and said that the cat had had a hard time and had enlisted the assistance of three other cats. One was digging holes for him, the second was covering them up and the third was way out in front opening up new territory. We probably need and should have two applied researchers for every one in basic research but we cannot do without the latter, the fellows who are out in front opening up new fields, developing new interpretations, new products, new ideas, and new methods. How few of the papers in our scientific journals are really original and new, in the sense that the theory of relativity, for example, is new? Yet one such good new idea is worth a thousand others because it stimulates a flood of new work.

Likewise one brilliantly original physiologist is worth a dozen less inspired workers. Physiology would profit much from an intensive Talent Search at the doctorate level. Personally, I must confess that I am thoroughly tired of the excessive publicity given in some of our journals to the Talent Search among the teen-agers. It is more important to seek out and reward the brilliant young investigator in his early 30's and to provide for every man of sufficient promise an unrestricted fellowship appointment where for once in his life at least he can give free rein to any original talent for research which he may possess without any regard to the practical applications of his efforts.

As more and more money is spent on research, more and more must also be spent on the administration and organization of that effort. More and more committees are set up in Washington, some of which overlap to varying degrees. There is a

Physiology Study Section in the National Institutes of Health; there is a Physiology Panel in the Office of Naval Research; there is a Physiology Panel under the Research and Development Board. There are committees on Aviation Medicine and Undersea Warfare in the National Research Council; meanwhile other panels on these same two subjects are being set up under the Research and Development Board. I do not say that there is no need for all these committees and many others which I have not mentioned but the very existence of so many physiological committees is proof of the fact that physiology like so many other sciences has at last taken to Horseback.

All of this takes the time of the men who serve on these committees. The sole object of research of the member-professor is in danger of becoming only the search for his last travel orders and his last Pullman stub. When he tries to sit by the fire and think, it is not to worry about why potassium is inside and sodium outside the cells but to consider how best to catch his next train. Or perhaps he is trying to foresee the practical problems which will suddenly be presented for solution to the Committee on Medical Research of some new OSRD at the outbreak of a still hypothetical but all too possible World War III. For these are the problems which should be undertaken now, in peace time, before it is too late. Anyhow he has too many diversions and rolling stones gather no moss. Such are the dangers.

How then shall we sum up the present situation with its promise of great progress and its subtle dangers? Do we want it as it is or must we change it? Admiral Groesbeck had an answer to this question in the form of a story which he told recently at a banquet of the NRC committee on Aviation Medicine. He lost his way, he said, on an automobile trip in the Southern mountains and stopped at a little cabin for some water. While the woman was pumping the water for him one of the many little children about the place rushed in and said, "Ma come quick, Johnny has fallen into the mud in the pigpen and he's sinking fast." All rushed out accordingly to the pigpen where, sure enough, Johnny's head was just visible above the mud. The mother hesitated only a moment, then planted her foot with firm decision on the boy's head and pushed him out of sight into the mud. Then she

brushed off her hands and returned to the pump remarking by way of explanation to the astounded Admiral, "It's easier to get another one than to clean him off."

I think we will all agree that it is easier to clean off the present situation in physiology than to get a new one. The baby-getting technique does not work for physiology. The American Physiological Society is dedicated to physiological research and the process of 'cleaning it off' involves chiefly the responsibility of taking care that physiology keeps its escutcheon bright and untarnished in spite of the corroding influences of the Horseback Era. In this effort, however, we are on the two horns of a dilemma and it takes a strong head to carry them both. On the one hand the Men on Horseback have honored us by asking for our services and we must not let them down. On the other hand we must not forego altogether the productive peace and quiet of the Ivory Tower. Mr. Truman said correctly that the scientist did not want to work in an Ivory Tower. Not exclusively so, anyway.

Every laboratory can easily handle a few problems in applied research. I heard of one university which offered to undertake one applied research problem at a cost of over \$60,000 when in my opinion a sufficient answer to the specific question proposed could easily have been obtained for \$3000. Perhaps the high price proposed was a measure of the distastefulness of the problem and perhaps it was to be used to pursue the problem into abstract and theoretical areas not really demanded in the original request. Anyhow this seemed to me unnecessarily defensive; or are we just 'money-mad'? Applied problems are not all bad nor do the basic problems turn out to be all good. Many are complete failures. Also many of the applied problems of the war have proved to be very stimulating and inspiring and have opened up extensive new areas for study. Moreover the Service laboratories for applied research are in most instances very fine places to work, with many very real advantages in the way of superior and unique facilities. In a recent tour which I have just completed of many of the military medical research installations of the Northeastern area I found considerable opportunity for the initiation of original studies and for the pursuit of these investigations in a thorough and basic manner. Much of our best physiological

work does and will, I trust, continue to emanate from these laboratories.

Finally I might confess that I have greatly over-emphasized the distinction between applied and basic research. The danger is rather from bad research as compared to good research. *Bad research on a basic problem is worse than good research on an applied problem.* In these days of easy money for grants-in-aid there is a danger of setting up so large a team of inexpert workers that the accumulated data exceed the digestive powers of the responsible investigator. The result is one type of bad research. The investigator over-busies himself to such an extent that he leaves himself no time to think. He is a victim of the project complex. He is an empire builder. He should get off his horse and go back to his knitting in the Ivory Tower.

I do not say that all extensive projects are bad. Most are necessarily large and very good. But the danger does exist. Another danger of easy money is the one-more-technician complex, the idea that any problem can be worked out by adding one more technician without additional allowance for time to think. Easy money and the empire-building complex is partly responsible also for the manpower shortage. In one famous case *A* helped *B* to obtain a fat grant. Then *B* in his affluence tried to take two skilled electrophysiologists away from *A*'s laboratory to help him on his project. One government agency creates so many new jobs by grants that other government agencies are unable to secure needed personnel. Salary levels rise, which is good, but the overall quality and quantity of good research does not. Thus we have a rising tide of diversion from true scientific work.

Like every serious problem this one deserves wise and rational consideration quite unlike the reasoning shown by two men who were out fishing in the ocean and found their boat rapidly being carried out by the tide, in spite of their strenuous efforts to row. They prepared to throw out the anchor but found the anchor rope missing. It seemed hopeless but there was no other solution. In desperation Erastus said, "Better throw it out anyway, boss. It might do *some* good."

It might do *some* good to refuse to engage in applied research. It might do *some* good to refuse to serve on government committees which eat up valuable study hours, however important and in-

teresting they might appear to be. It might do *some* good to refuse approval of large grants-in-aid which merely create new jobs and rob Peter to pay Paul. It might do *some* good to kill the goose that lays the golden eggs.

But it would also do harm and would be as futile as an untied anchor. The one sure anchor with a sure anchor rope lies in any measure which will entice the best possible brains into physiology and give to each man in both Service and university laboratories the best opportunity to develop his individual potentialities for original work. Quality attracts quantity and the young men in physiology today are 'red hot'. I should hate myself to be in

competition with the 1948 crop. The war has shown these young men that physiology is useful and that a good man can accomplish big things in that field. It is as if gold has been discovered and a young gold rush has started. (I refer to scientific gold here—not the stuff that glitters.) There should soon be enough good men to dig holes and cover them up as well as to look for new places to dig. Thus physiology is stronger today in the horseback era than ever before and it will continue to grow if we provide good men and continue to feed them calories in the form of good salaries and plenty of vitamins in the form of scientific freedom.

# MUSINGS OF A PHYSIOLOGIST<sup>1</sup>

MAURICE B. VISSCHER

*President, The American Physiological Society, 1948-1949*

LAST YEAR we had the rare pleasure of listening to the whimsical treatment of the topic 'Physiology on Horseback' by my respected predecessor Wallace O. Fenn. If he had done nothing more than to record for all time his fable of the sick cat and basic research, his speech would have been worthwhile. Of course in saying so, I am not deprecating the value of the other gems he cast before us.

At this juncture I am reminded of the definition of education which has been propounded by my friend Herbert Feigl, the philosopher. In describing contemporary education he says that it consists in the casting of artificial pearls before real swine. I have been told on what I consider to be excellent authority that the interest of the Congressional House Committee on UnAmerican Activities in the investigation of text books used in American colleges and universities was aroused by a secret report that some teachers were instead throwing real pearls around. I cannot vouch for the accuracy of this story because such matters are strictly confidential and I was told it by the mother-in-law of a University President who said she heard it from her cleaning woman who is the wife of a janitor in the philosophy building, who said he had read it in the waste paper he picked up from the office of a graduate student. However, it has the ring of truth about it. My only reason for doubting it is that no ex-communist, ex-editor of *Time*, *Life* or other such lucid publication has found evidence for it in his Halloween pumpkin. However, that may turn up ten years from now.

Wallace Fenn put me in a very bad spot by giving the sort of speech he did last year. It would be unfair to him to say that we had not expected him to come out with a model of the witty after-dinner speech. As one who has worked with him in nu-

merous activities for a dozen years I have to admit that I knew he was capable of doing exactly what he did, but, to be honest, I must say I thought that he was much too generous to do it. He knew perfectly well that he was setting a standard that the rest of us who are following along in the President-Elect, President and Past-President groove might not be able to approach, let alone equal. However, the job is now awaiting me and I must not waste your time with trivialities.

'Physiology on Horseback' was a title, however, that permits one to choose as to whether the next year one will keep up with the horseplay or go into something more consonant with the staid dignity of academic mores. I have thought about variations on the horseback theme. I have wondered whether I should not perhaps discuss as a physiologist the relative merits of riding English or Western saddle, but since I ride English and live in what some provincial people in the East think is the West I felt I might be in some confusion. Then it occurred to me that I might simply take the original topic and discuss it in full. Professor Fenn, as you may recall, did not say anything that really bore on his title. It was simply a jumping off place and allowed him to tell some jokes with a tinge of barnyard odor, without having it seem incongruous. Not to break the tradition I hope to bring in a bit of the pungency of the lower alimentary tract of the higher vertebrates, but I shall, like my distinguished predecessor, keep the concentrations of the indoles, skatols and mercaptans down to respectable limits.

Not being a psychoanalyst I am not prepared to say why our former President chose the title Physiology on Horseback, but I am sure there must be some deep, dark secret, perhaps dating from his intra-uterine past, which accounts for it. For my part I have tried to choose a topic which can have no possible psychoanalytical, or shall I say irrational, interpretation. That is why I have chosen to speak without any announced subject. This way

<sup>1</sup> Past-President's address, read at the dinner of the American Physiological Society, at the Second Fall Meeting, Augusta, Georgia, September 16, 1949. Published at the request of the Council.

I enter into no contract, real or implied, except to occupy some of your time. This I hope to do without boring you, but I make no promises. Since I have lived for more than thirty years in a so-called higher educational atmosphere I have had little exercise in humor, other than that which I could see in the position of academic people in a generally suspicious and, by turns, contemptuous and jealous world. However, our academic colleagues have for the most part been able to retain a sense of humor, as something more than a vestigial remnant of an organ. They are, I believe, too much restrained in their use of humor, however, particularly in meeting the problems that arise in connection with the emergence of science as a Frankenstein monster which the bewildered people of the world do not know what to do with, and react by suggesting that the scientists who created the beast should either be eliminated or at least sterilized to render them incapable of other acts of procreation like it.

I wonder whether scientists might not get further in their approach to the Congress and State Legislatures if they were to concentrate on letting the American people know how ridiculous certain U. S. Senators appear to them rather than by taking their uniformed prattle about nuclear physics seriously and acting as though it came from a rational mind. One devastating turn of ridicule would have more effect than a thousand pages of reasoned argument in the rough and tumble of a political debate.

A few months ago I listened to one of the Deputy Surgeons General of the U. S. Public Health Service explain the underlying principles of the theory of crime and guilt. He told the story of a chemist in England who was hauled into court charged with illegal possession of a still. The chemist admitted possession of the still but he couldn't understand why it was illegal to have one. He was told that it was because of what he might do with the equipment in his possession. He then became very much concerned and said he was ready to plead guilty, but he wanted also to plead guilty to other crimes, in particular that of rape. He was asked whether he had actually committed rape. "No", he said, "but I have the equipment for it in my possession!"

It occurs to me to suggest that those people in the House Committee on UnAmerican Activities

who purport to see spies behind every laboratory door are perhaps suffering from one or more of several difficulties. Either they are confused, as was the English chemist, with the distinction between possession of an organ, in this case the brain, and the use of it for immoral purposes; or they may themselves have so little intelligence that they are unable to conceive of a rational basis for loyalty to one's country; or their own standards of ethics may be so low that they cannot comprehend that most people are just naturally decent, kind, honest and reliable.

I am afraid that I couldn't assemble the kind of positive proof that would satisfy the Editorial Board of the *American Journal of Physiology* as to the statistical reliability of the conclusion, but it is my everyday experience that most people I come in contact with are helpful, reliable and kind. I have found this to be true in a dozen countries and in persons of every race. The only marked exceptions to this rule arise when men ride in motor vehicles, when they get into direct competition for privileges of one sort and another and especially when they get into professional politics.

I do not propose to publish these conclusions as scientifically verified facts but I would be willing to stake my reputation as an observer of human behavior on their general validity. Consequently, I am much more convinced of the general decency of the average citizen than I am of the average politician. A few months ago a newly elected U. S. Senator said to me in private conversation, "It has been a great shock to me to see at first hand how few of our Congressmen speak their own minds or vote their own principles, in fact how few of them have any real principles at all. What pass as principles are dictated by pressure groups and powerful sponsors."

About a month ago there appeared in *Science* a summary of a report by the A.A.A.S. Committee on Civil Liberties for Scientists, of which Committee I had the privilege of serving as the Chairman. In connection with the preparation of that report I had an opportunity to see how perilously close we are in this country to a total eclipse of intellectual freedom. Fear and hatred have so stirred up the midbrain and hypothalamic centers of millions of otherwise sane people that they no longer retain cortical dominance.

It has been impossible for me to refrain from thinking about the reasons for a special distrust of science and scientists by many people. First and foremost among the reasons for public suspicion of the loyalty of scientists is, I believe, the fact that almost all scientists recognize that science itself is universal and has no uniqueness for any race, religion or nation. The general acceptance by scientists of the thesis that basic knowledge must flow freely across political, geographic and racial lines if the national welfare is to be best served, causes many well-meaning people to doubt that such persons could possibly be loyal nationals in any country. Such a doubt, however, betrays a remarkable lack of comprehension of the realities of the situation as well as of human nature. In arguing the advantages of free exchange of scientific information the scientist is simply saying that any other policy is ultimately stultifying to scientific progress itself and that it would soon lead to equality rather than superiority of scientific and technological status in one country as compared with other nations. The more nationalistically selfish a scientist is, the more vigorously he should argue for absence of secrecy in basic science. The average Congressman cannot, perhaps, be expected to comprehend such reasoning. Not because he is too stupid, I believe, but rather because he knows too little about the facts of the case and he is so busy worrying about his re-election that he has no time to waste on such abstruse matters as what makes for scientific supremacy in a country.

The persons who worry about the loyalty of scientists also grossly misjudge human nature. They seem to assume that scientists are fools of the first order. We all know that scientists are fools in the eyes of those who put financial return above other rewards in life, but this is really only second-degree foolishness. A first-order fool is one who makes his position in society untenable by what he does. He steals from a bank, commits murder or betrays State secrets. I have scarcely known any scientists who gave evidence of being first-order fools. I doubt that there are many. Despite a lot of press releases on the subject the House Committee on UnAmerican Activities hasn't been able as yet to find a single one in this country.

The second reason why scientists are suspect, I believe, is that they are ordinarily believed to be

idealists, and idealists are thought to be dangerous. To dispel this notion I propose that scientists should demand more monetary compensation and do it more vigorously. This should disabuse anyone of the notion that scientists are peculiarly lacking in worldly wisdom and expensive tastes.

A third reason for distrust of scientists as a class is unquestionably because they are considered to be intellectuals. From the time of Socrates or before the man of reason has always been more or less suspect to his dominant contemporaries. In our times the opprobrium attached to the term 'brain-trust' portrays the unwholesome significance attached to cerebral activity, by a large and powerful segment of the American population. Anti-intellectualism is a hallmark of mid-twentieth century America. Thinking entails questioning, questioning implies lack of faith, lack of faith means rejection of authority, and rejection of authority is repugnant to those in positions of authority. Therefore, intellectuals are very dangerous people.

Of course that conclusion is correct, as far as authoritarians are concerned, but not as far as reasonable people who want to learn the truth are concerned. The dogmatists in America, in Russia or in religious sects are bound to fear enlightened reason, but for the simple man reason is nothing to be feared, but rather something to trust for his betterment, if it can be given free play.

The situation for scientists in our current world is not a happy one because science has become a major factor in international power politics. Scientists have become pawns in a planetary chess game. The pawns are being sacrificed to save the major pieces. History tells us that civilizations do not have immortality. Great Powers have risen and fallen. Cultures have waxed and waned. The human race, however, has always managed to survive and I predict that it will do so again, even in the face of atomic and biologic warfare. I do not, however, care to risk a judgment as to how much of any present major world power will survive.

History also tells us that since the invention of the printing press knowledge has survived every national catastrophe and we may rest quite well assured that science as a body of knowledge and as a discipline will survive the new stupidities which our generation or one of the next may very possibly commit. Nevertheless we have no cause to be

supinely silent while atrocities against decency and reason are being committed. While there is still freedom to protest, enlightened persons have an obligation to point out the stupidity of the suicidal urge of dominant groups to suppress the development of science.

It is a fetish among many contemporary pseudo-intellectuals, as I presume it has been from time immemorial, to attempt to build up a logical case for believing that what is quite obviously true is false. For example, we live in a civilization in which practically everything we use for sustenance, for pleasure or for enlightenment is in one way or another the product of, or conditioned by, the results of scientific discovery. Yet every day our newspapers, our magazines, our new books, our preachers and our politicians are trying, many of them, to argue that it is not true. They would have us believe that these facts are in essence illusions. We are told that science is the destroyer of ancient virtues and that it has created a world in which man has so much power over nature that he cannot be trusted in exercising his reason in controlling it. We are told that only by return to authoritarian religion sustained by an elite power group of one sort or another, depending for its color upon which side of the East-West line one happens to be, can a sensible and just societal system be achieved.

As scientists we are committed to rejection of any preconceived opinion save one. Our dogma is that the closest approach to truth can be achieved by the scientific method. Such a commitment is the common bond between all true scientists. It links all scientists in the struggle against the bigotry of anti-evolutionists in the United States and Lysenkoism in Russia. It should also link us all in the current struggle for preservation of scientific freedom from politics in the United States. Some of you who have not been intimately concerned with the civil liberties problem may feel that I am over-emphasizing the gravity and imminence of the present danger. Certainly we as scientists should avoid even the suspicion of paranoia in the present situation. Some group must keep its head clear. But in escaping paranoia we need not feign blindness. I recommend that scientists, as individuals and groups, reject every proposed encroachment upon their freedom of thought. They should treat the anti-intellectualism of our times as one would a

carcinoma. There is always danger from the invasiveness and the metastatic tendencies of the tumor. Many persons die of cancer, but a few are cured. Our civilization may die, too, of the cancer invading it, but there is a chance for cure. In any case there is no sense either in complacency or hysteria. My prescription for the disease besetting us in eradication of the primary lesion of anti-intellectualism and a vigorous campaign of general enlightenment to try to prevent fatal metastatic spread. I am, as you can see, hoping that general enlightenment may play the part of x-ray irradiation in the analogous body cancer. Then, like good physicians, we should exude a generous amount of optimism, warranted or not, because live or die the patient deserves some solace.

When I started this speech I told you that my title was chosen to allow me to touch on anything without straying from my subject. I have at least lived up to my implied promise not to confine myself to a single idea. A good speech is, of course, something quite different. In it, as you know, one says what he is going to say, says it in two or three different ways, reminds the audience of what one has said, and sits down amid applause. I never intended to give you a good speech tonight because I was sure you would be bored by it. The truth is I simply wanted to get a few ideas off my chest and I thought a Past-President was in a good position to say what he pleased with impunity. Immunities of this type come only once in a lifetime. Furthermore they are not permanent. So I am counting on my protection lasting only until next spring when you will have another Past-President whose vagaries you will also have to tolerate on an occasion like this. I realize moreover that one cannot go too far in this matter of immunity. There is a hyperimmune state and there is allergy, not to mention anaphylactic shock. I am not sure just which state is more likely to be produced by an overdose of past-presidential antigen, but they are all undesirable. So to protect you against a shocking dose I propose to administer an antispasmodic and a soporific.

In thinking about the happy state of the Past-President I was reminded of a very impressive occasion just a little more than twenty-three years ago. I happened to be spending the year at University College in London with Professor Starling

at the time that a testimonial dinner was given in his honor, and more or less incidentally in honor of Dr. G. V. Anrep who was leaving to go to Cambridge the next year. The speeches in honor of Starling were all of a very high order—everything was said that should have been said in honoring one of Britain's greatest scientists. They were exactly in proper taste and consequently I have forgotten them completely. However, with respect to Anrep the honors were done by Professor A. V. Hill. He handled the matter quite differently. He mentioned all of Anrep's idiosyncrasies, such as his collecting tendencies. Apparently whenever apparatus was missing in any laboratory at University College, everyone's first thought was to look in Anrep's rooms. Usually it was recovered in that way. Hill went on to say that in preparation for this speech he had taken a holiday in Cornwall and in search of the right thing to say he had gone into a number of churchyards reading inscriptions on tombstones. One that he saw he said seemed to provide the clue to the proper words. It read, "Here lie the bones of John Jones. In his life he knew several respectable people." Hill went on, "I think we should put a plaque on the door of Anrep's lab after he leaves us, and inscribe on the plaque: 'In this room for many years worked G. V. Anrep. During his stay here he was associated with several respectable physiologists'."

To relate this to past-presidents I have wondered whether the Society ought not present each of them with a scroll when they leave which might read somewhat as follows: "In this office served blankety blank blank. During his tenure he served a thousand highly selected physiologists." I make this suggestion because past-presidents, like worn-out shoes, are apt to be discarded pretty lightly. Some fitting recognition of their good intentions and hard labor, even if their accomplishments have been minimal, will keep their spirits up during their declining years.

Finally before I sit down I want to say to you, my colleagues, that I am really very happy about two things. First, I am pleased with the honor and opportunity of having been allowed to serve as Councilor, Secretary and President of this Society, because I believe that the American Physiological Society is playing a highly constructive role in promoting scientific research and teaching in physiology on this continent. I am also very pleased by the opportunity I have had to work on various projects with many of you because it has allowed me, I hope, to call many more of you friends instead of mere acquaintances, then would otherwise have been possible. I appreciate both the honor you have done me and the trust you have placed in me.

# ABSTRACTS OF PAPERS READ 1949 FALL MEETING

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*An asterisk following an author's name denotes "by invitation"*

*Functional relationships between the middle and posterior ectosylvian areas in the cat.* HARLOW W. ADES. Emory Univ. School of Medicine, Atlanta, Ga.

The primary projection area of the auditory system in the cat lies across the superior part of the anterior middle and posterior ectosylvian gyri as indicated by electrical response to auditory stimulation. The inferior portion of the posterior ectosylvian cortex under favorable circumstances also shows a response similar in general characteristics to that of the primary projection area, but having a much higher threshold and lower amplitude. Also, the latency of the inferior postectosylvian response is always longer by 3-5 msec. The inferior postectosylvian response can be augmented by strychninization of the superior part of the middle ectosylvian cortex. Furthermore, under conditions of repetitive stimulation with clicks, the postectosylvian response is paced by the midectosylvian activity which in turn is characterized by strychnine-augmented negative phase paced by the stimulus. Strychninization of the postectosylvian area results in randomly recurring strychnine spikes in this area which are not related to the stimulus and which do not fire the primary midectosylvian area. These results are taken to indicate that the inferior part of the posterior ectosylvian gyrus is dependent on the primary auditory area for its specific activation and constitutes a secondary area in the sense of an association area.

*Influence of acute epinephrine hypertension on calculated resistance of canine femoral vascular bed.* RAYMOND P. AHLQUIST (introduced by W. F. HAMILTON). Dept. of Pharmacology, Univ. of Georgia School of Medicine, Augusta.

Flowmeters such as the rotameter or bubblemeter when used to measure arterial inflow delay the arrival of intravenously injected substances in the periphery by the time necessary to displace the blood in the flowmeter system. There is, therefore, a short period of time during which the measured bloodflow is under the influence of only the central vascular actions of the substance. When the femoral arterial flow of anesthetized dogs is measured by one of these meters, it will be found that during the acute pressor response to intravenous epinephrine the flow will increase at a

faster rate than the pressure. This will result in a decrease in the calculated resistance,  $P/F$ , until the epinephrine passes through the metering system, reaches the femoral vascular bed and produces its characteristic constriction and increase in resistance. An analysis of this effect using a device to further delay the appearance of the epinephrine in the periphery and using also cross-perfusion into a recipient leg shows that the fall in resistance is due to two separate factors: 1) vascular distention as described by Green *et al.* and 2) neurogenic reflex dilation resulting from the increased arterial pressure.

*Localization in anterior lobe of cerebellum of dog, with special reference to the placing reactions.* G. M. AUSTIN, W. W. CHAMBERS, AND W. F. WINDLE. Harrison Dept. of Surgical Research and Dept. of Anatomy, Univ. of Pennsylvania School of Medicine, Philadelphia.

Following lesions of the interpositus nuclei, and after ablation of the anterior lobe of the cerebellum of cats, Chambers and Liu describe loss of the placing reactions. The present study was carried out on 25 adult dogs in which selective ablations of the anterior lobe and other control areas of the cerebellum were done. The animals were killed after survival periods varying from one week to 5 months. During this time the animals were frequently tested for station, gait and the status of the placing and hopping reactions. Following localized lesions of culmen or centralis, specific defects in station and gait of the fore and hind limb respectively were noted. These consisted of high stepping, circumduction and abduction of the limb. There was also a complete loss or marked diminution of the contact placing reactions and to a lesser degree the hopping reactions in the corresponding limb. Localization tended to show slight overlap so that the hind limb was involved in lesions of the caudal two-thirds of centralis and rostral border of culmen, forelimb with lesions of the caudal two-thirds of culmen and rostral border of simplex. The precise effect of nuclear lesions awaits the histologic study of serial sections.

*Effect of interruption of blood flow to stomach for varying periods on potential difference and rate of secretion of HCl.* F. J. BAJANDAS\*, FRED E. COY, JR.\*, THOMAS

P. DEGRAFFENRIED II\*, AND WARREN S. REHM. Dept. of Physiology, Univ. of Louisville School of Medicine, Louisville, Ky.

In an attempt to find out more about the role of the potential difference across the stomach in the secretion of HCl, the relationship between these characteristics was studied during and following periods of interrupted blood flow. A portion of the amyotomized dog's stomach was placed in a lucite chamber. Histamine and mecholyl were used as gastric stimulants. Clamping the pedicle (for periods of from 2 to 55 minutes) to the stomach resulted in a decrease in both the P.D. and secretory rate. The percentage decrease in secretory rate was in every case greater than the percentage decrease in P.D. When the blood supply was re-established the P.D. rapidly increased and overshot before returning to its original level. Following clamping of the pedicle for periods of about 15 minutes or longer, there is a marked and prolonged overshooting which is associated with a zero secretory rate. As secretion is re-established the P.D. falls to the level of the secreting stomach. In the resting stomach the overshooting of the P.D. is practically absent. It is suggested that the marked overshooting in the secreting stomach is a result of the wiping out of secretion. The rate of decrease of the P.D. following clamping of the pedicle is modified by the oxygen tension of the saline in contact with the mucosa. Replacing areated or oxygen-free saline with oxygen-saturated saline resulted in an increase in P.D. within about one minute, which is evidence that the EMF's originate near the mucosal surface.

*Effect of pain on electroencephalogram.* F. B. BENJAMIN\* AND A. C. IVY. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

In a series of 17 experiments on human subjects an attempt was made to learn whether pain produces any specific changes in the electroencephalogram. Frequently an attention wave occurred on the first application of the pain stimulus. In addition to this there was a decrease of amplitude in the nature of a loss of electrical entropy. The latter changes were bilateral, even on unilateral stimulation and they were noticed in all large cortical areas but were more pronounced in the parietal, occipital, temporal and frontal lobes in descending order. These pain effects vary little with change of intensity or nature of the pain stimulus and are similar to those produced by cold, heat and touch. Bipolar registration, coupling 2 to 8 electrodes, needle electrodes imbedded in the sphenoid bone, other retropharyngeal electrodes and needle electrodes applied to the base of the skull in the region of the foramen ovale did not produce any essential improvement as far as pain registration is concerned. In 4 experiments with

morphine it was found that the flattening of the middle range of waves which is produced by both pain and morphine was so similar that it was not possible to make any reliable interpretation of the recordings.

*Observations on progressively reduced nervous system in cats.* DONALD S. BICKERS, ERIC W. PETERSON AND JEAN SCHERRER. Dept. of Psychiatry, Univ. of Illinois College of Medicine and Illinois Neuropsychiatric Institute, Chicago.

Progressive reduction of the nervous system by supra segmental ablations of component parts results in regular (reproducible) syndromes which clarify the functions of remaining parts and interrelationships between all components. During prolonged observation, changing immediate postoperative findings yield to relatively stable states of lower functional capacity. Decortication, sparing the rhinencephalon, results in a sluggish blind cat capable of stereotyped behavior, with extensor hypertonus in suspension and diminished positive support. Anterior cerebellar lobe ablation alters this syndrome chiefly in postural mechanisms evidenced by neck retraction and increased extensor hypertonus. Trunk righting, sitting, standing and walking are rendered difficult or impossible by antelexion of the lumbosacral spine and thighs (pelvic jackknifing). Complete decerebellation alters the decorticate syndrome similarly but more profoundly. During prolonged stereotyped righting attempts, the forequarters are occasionally righted. Dysmetria, intention tremor and dyssynergia are absent; stereotyped movements are well performed. Cerebellar ablation alone permits initiation of complex motor activity but impairs performance. Dyssynergia, dysmetria and weaving 'tremor' of the head predominate. The last is eliminated by fixing the shoulders. Anterior cerebellar lobectomy alone results in postural alterations (neck retraction, extensor hypertonus with pelvic jackknifing), dysmetria and dyssynergia. Standing and walking are impaired or lost; scratch reflex is normal.

*Neurophysiological applications of automatic anesthetic regulator controlled by brain potentials.* REGINALD G. BICKFORD. Laboratory of Electroencephalography, Mayo Foundation, Rochester, Minn.

Studies in this laboratory have shown that in the animals investigated (rabbit, cat, monkey and man) a simple and characteristic relationship exists between depth of anesthesia produced by barbiturate drugs or ether and the integrated potential output of the cortex. The latter is derived by electrical integration of the amplified electroencephalogram. The constancy of the above relationship suggested the possibility of using brain potentials to control the dosage rate of the anes-

thetic so as to achieve automatic administration. The apparatus uses a pentode as an integrator tube to convert the amplified brain potentials into a unidirectional current which charges a storage condenser. Discharge of the storage condenser at a predetermined voltage energizes (via a thyatron circuit) a stepping relay the threaded shaft of which is geared to the syringe containing the anesthetic mixture. The latter is connected to a vein or to a vaporizer according to the kind of anesthetic in use. When this system is in operation anesthetic is fed to the animal at a rate which varies directly with the integrated potential output of the cortex. Since the latter decreases with deepening anesthesia an equilibrium point is reached and maintained indefinitely at a depth of anesthesia which can be controlled by the circuit adjustments. The presence of a feedback cycle gives the system homeostatic properties and accounts for the smoothness and accuracy with which anesthesia is maintained. Anesthesia has been maintained automatically without any circuit readjustments in animals for periods of 2 or 3 days. Successful automatic control of anesthesia has also been achieved in the human being.

*Muscle glycogen fractions.* WALTER LYON BLOOM, GEORGE T. LEWIS AND G. C. KNOWLTON. Depts. of Biochemistry and Physical Medicine, Emory Univ. School of Medicine, Atlanta, Ga.

This report is concerned with studies of the alkali-soluble and trichloroacetic acid-soluble fractions of rat muscle after denervation and during reinnervation. Determinations on both gastrocnemius muscles of rats were made at daily intervals on 12 successive days, starting 12 days after the crushing of one sciatic nerve at the level of the great trochanter. The muscle of the intact leg served as the control for each animal. The glycogen content of denervated muscle was decreased to approximately 50 per cent of its control value from the 12th to the 17th day, confirming the observations of Hines and Knowlton. Both glycogen fractions decreased in the denervated muscle. On reinnervation these values increased suddenly to normal or supernormal values. This rather abrupt increase was noted within a 48-hour period, from the 18th to the 20th day following denervation. However, the weight of the denervated muscle continued to decrease slowly throughout the period of observation (24 days following crushing of the nerve). The control values for this series confirm the previous findings of Bloom, Lewis and Schumpert of the presence of two glycogen fractions in rat muscle, one of which is soluble in 10% trichloroacetic acid as well as in strong alkali and makes up about 50 to 55% of the total alkali-extractable glycogen. These studies show that both fractions are affected by de-

nervation and reinnervation and more accurately limit the time of glycogen restoration during reinnervation.

*Influence of thyroid and adrenals on protein breakdown in eviscerated rats.* PHILIP K. BONDY (introduced by JAMES V. WARREN). Dept. of Physiological Chemistry, Yale Univ. School of Medicine, New Haven, Conn.

The effects of adrenalectomy, adrenal cortical extract, thyroidectomy and thyroxin upon the plasma amino acid nitrogen have been studied in eviscerated rats. Following removal of the adrenal glands, there was a reduction in the rate of rise of the amino acid concentration in the plasma of eviscerated rats. This effect was corrected by the administration of 0.1 ml. of adrenocortical extract/100 gm/hr. for 3 doses. This dose of adrenocortical extract was without effect in rats with intact adrenals; however a dosage level five times as high produced a significant rise. This rise could be abolished by the simultaneous administration of 0.5 gm. of glucose i.v. Thyroidectomy also caused a reduction of the rate of rise of plasma amino acids. The effect was independent of reduced adrenal activity, since 0.1 ml. of adrenal cortical extract failed to correct it. Twenty micrograms of thyroxin daily for one week, however, restored the rate to normal. These observations indicate that the protein-catabolic effects of the adrenal cortex do not require the presence of the liver for their activity, and therefore are not primarily associated with alterations in the rate of urea formation; and that they can be antagonized, in the absence of the liver, by adequate amounts of glucose. The thyroid protein-catabolic effects are independent of the adrenal gland.

*Reflex orthostatic dyspnea associated with pulmonary hypotension.* HOWARD B. BURCHELL, H. FREDERIC HELMHOLTZ, JR. AND EARL H. WOOD. Mayo Clinic, Rochester, Minn.

A patient with a post-traumatic intrathoracic venous-arterial type of blood shunt exhibited approximately a three-fold increase in ventilation whenever he was in an erect position. Repeated studies showed little variability in the ventilation increments with graded postural changes. The increase in ventilation occurred within a few seconds after assumption of the erect position, consisted mainly of an increase in the tidal air and persisted with a uniform respiratory breathing pattern irrespective of an increase in  $pH$  of the arterial blood (such as 7.46-7.54). The ventilation returned to its previous value within a few minutes when the subject was returned to the horizontal position. The systemic blood pressure showed no significant changes when the patient was tilted. The oxygen saturation of arterial blood with the patient in the supine position varied

between 83 and 90% and decreased approximately 15 points when the patient was erect. Cardiac catheterization revealed a very low right ventricular (9/2 mm. Hg) and pulmonary arterial (7/1 mm. Hg) pressure. When the subject was tilted to a 60° slope, the systemic blood flow decreased from 5.2 to 4.4 l/min. and the venous shunt increased from 44 to 51%. The patient's breathing of 100% oxygen at ground level or in a pressure chamber at increased pressures up to 4 atmospheres, in the latter instance the arterial blood being normally saturated, did not prevent an increased ventilation in the semierect position. In the teleoroentgenogram the distance from an assumed level of the pulmonary valve to the lung apex was 17 cm. It is suggested that the dyspnea was initiated by the pulmonary reflex related to the pulmonary hypotension.

*Application of Dreywood's anthrone reagent to determination of inulin in plasma and urine.* WALTER H. CARGILL (introduced by JAMES V. WARREN). Dept. of Physiology, Emory Univ. School of Medicine, Atlanta, Ga.

Existing methods for the determination of inulin are based upon hydrolysis by prolonged heating in the presence of acid and subsequent photometric determination of the resulting fructose. The degree of hydrolysis is dependent upon the duration of heating and is difficult to control with accuracy. Unstable colors which fade rapidly on standing are produced by both diphenylamine and resorcinol. The present method was designed to obviate these sources of error. Glucose is rendered non-reactive according to the method of Little *et al.* (*Federation Proc.* 8: 98, 1949). To 3.0 cc. of a zinc sulfate filtrate in a large test tube add 1.0 cc. of 4N NaOH, cover with marbles and immerse in boiling water for 10 minutes. Cool and add 1.0 cc. of 4N H<sub>2</sub>SO<sub>4</sub>. Ten cc. of anthrone reagent, prepared as described by Morris (*Science* 107: 254, 1948), are then added rapidly from a broken-tip burette. The solutions are mixed by swirling, allowed to stand at least 10 minutes, and read in a photoelectric colorimeter at 620 mμ. The color produced is stable at room temperature for several hours. The reaction is quite sensitive, it being possible to determine with accuracy final concentrations as low as 0.1 mg. %.

*Acclimatization of mice to carbon monoxide and low oxygen.* ROBERT T. CLARK, JR., ARTHUR B. OTIS AND S. WAH LEUNG. Dept. of Physiology, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Mice were exposed to carbon monoxide in gradually increasing concentration from 0.03% to 0.15% over a period of 14 days, after which the animals were re-

moved to room air for a day. They were then tested for tolerance to a simulated high altitude of 34,000 feet. Survival times of the mice previously acclimatized to CO were considerably longer than mice unacclimatized to CO. The converse of the above experiment was attempted by subjecting mice to an atmosphere containing oxygen in gradually decreasing amounts from 10% to 7% for a period of 14 days. The controls and experimentals were then exposed to an atmosphere containing 0.25% carbon monoxide. Survival times of the mice previously acclimatized to low oxygen were considerably longer than the mice unacclimatized to low oxygen. Blood studies were made on mice after acclimatization to carbon monoxide and low oxygen. In both cases the oxygen capacity increased about 60% above the controls, and the hematocrit 85%. The carbon dioxide capacity of the plasma was found to decrease for the mice acclimatized to low oxygen, but showed no change from controls for these animals acclimatized to carbon monoxide. The pH of the blood of mice acclimatized to carbon monoxide was found to be considerably higher than that of the mice acclimatized to low oxygen. The rate of oxygen consumption was the same the first few days of exposure to carbon monoxide and low oxygen as at the end.

*Physiology and metabolism of flavonoid pigments ("Vitamin P").* WILLIAM G. CLARK. Veterans Administration Center and Univ. of California School of Medicine, Los Angeles.

Methods were developed for the quantitative determination of flavonoids in biological materials and studies made of the absorption and excretion of several flavonoids orally administered to rats, guinea pigs and humans. Quercetin-6'-sodium sulfonate and esculetin-4-sodium carboxylate were absorbed and excreted in the urine of rats to the extent of 5% of the fed dose. None of the others were absorbed from the intestine of rats or guinea pigs, nor excreted in the urine or feces of humans, including rutin and its soluble complexes. Rutin was destroyed on incubation with human feces. Oral or parenteral treatment with several flavonoids had no effect on the hemorrhagic symptoms or survival of mice and guinea pigs with experimental thrombopenic purpura. Non-thrombopenic purpura was produced in dogs with anti-vascular endothelium serum but was impractical as a tool for such studies. The two flavonoids listed above as being slightly absorbed by rats had no effect when fed in the drinking water to chronically dicoumarolized rats with regard to hemorrhagic symptoms or survival. Flavonoids had no effect on bleeding time in guinea pigs, extravasation of dye into histamine skin wheals in rats, the LD<sub>50</sub> of intravenously adminis-

tered ficin in rats, and anaphylactic shock in guinea pigs. Rutin in doses over 100 mg/kg. I.P. increased the LD<sub>50</sub> of I.V. histamine in guinea pigs 1.5 times over the controls, and ten times less quercetin sulfonate increased it 2.0 times. All other flavonoids tested were inactive. Several flavonoids had a moderate but non-specific inhibition of the spreading action of hyaluronidase in rats, guinea pigs and rabbits. Scurvy markedly decreased the spreading effect and flavonoids did not alter this.

Analysis of reports on flavonoids indicates no direct, specific 'vitamin P'-like or drug effect, suggesting a non-specific stress. Quercetin sulfonate, 10 mg/kg. I.P. decreased the adrenal ascorbic acid content of intact rats as much as epinephrine or arterenol, which points to a pituitary-adrenal mediation. This suggests the use of ACTH and Compound E in hemorrhagic disorders and sensitization phenomena claimed to be favorably influenced by flavonoids and related substances.

*Effect of thymic extracts on neuro-muscular response.*

GEORGE A. CONSTANT\*, EUGENE L. PORTER, HERBERT M. SEYBOLD\* AND ARISTOPHANES ANDRONIS\*. Carter Laboratory of Physiology and Dept. of Neuro-Psychiatry, Medical Branch, Univ. of Texas, Galveston.

In a preliminary report, Constant, Porter, Andronis and Rider showed that the injection of thymic saline extracts (myasthenic as well as non-myasthenic) into the general circulation of the cat diminished the contraction height of the tibialis anticus muscle stimulated through its motor nerve. Using splenic extract as a control they found that it increased neuro-muscular response. The present report is a continuation of this work. At the present, 23 cat nerve-muscle preparations have been employed. The tissues were emulsified in 1:5 dilution of physiological saline for a period of 2 to 3 minutes. The emulsion was then passed through a glass wool filter, the filtrate being used in all determinations. The injections consisted of 5 to 10 cc. of the extract in question. Of 48 experiments with human thymic extracts, 43 resulted in decreased muscular contractions, whereas 5 had no effect. Six experiments each were performed with pooled dog thymic extract and pooled cat thymic extract. In the case of the dogs, 5 resulted in decreased muscle contractions and one showed no effect. In the case of the cats, 4 resulted in decreased muscle contractions. Then after the injection into the general circulation of 0.5 mg. Prostigmine, 2 experiments showed no effect. Using splenic extract as a control, 14 experiments resulted in 9 showing an increased neuro-muscular response, while 5 had no effect.

These observations seem to indicate that thymic saline extracts contain a curare-like substance.

*Explanation of quantitative difference in production of HCl resulting from directional application of current.*

FRED E. COY, JR.\*, THOMAS P. DEGRAFFENRIED II\*, F. J. BAJANDAS\* AND WARREN S. REHM. Dept. of Physiology, Univ. of Louisville School of Medicine, Louisville, Ky.

In previously reported work (*Am. J. Physiol.* 144: 115, 1945) it has been found that the application of electric current from serosa to mucosa of the secreting stomach results in an increase in H<sup>+</sup> production, and the application of current in the opposite direction a decrease in H<sup>+</sup> production. Current sent from mucosa to serosa is approximately three times as effective in reducing H<sup>+</sup> secretion as current sent in the opposite direction is in increasing H<sup>+</sup> production. An equivalent circuit has been proposed to account for this orientation of the P.D. across the stomach and the directional effect on secretion of applied current. This equivalent circuit is essentially an EMF directed toward the serosal side with a shunt across it. It is assumed that the current in the shunt controls the production of H<sup>+</sup> ions. Analysis of this circuit reveals that, if the parameters remain unchanged with the application of current, the change in current through the shunt should be numerically the same with the application of current in either direction. In the experiments reported here the open circuit P.D. was measured with a potentiometer during periods of current flow by momentarily breaking the circuit. It was found that with current sent from mucosa to serosa there was a relatively large decrease in the open circuit voltage, while with currents sent in the opposite direction there was a much smaller change. It is possible, on the basis of these findings, to offer a tentative explanation of the quantitative difference in the directional effect of the current.

*Variability in blood pressures of laboratory dogs.*

F. N. CRAIG AND JOHN A. ZAPP, JR.\*. Applied Physiology Section, Medical Div., Army Chemical Center, Md., and Haskell Laboratory of Industrial Toxicology, E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.

This is a study of 57 laboratory dogs by one author, J.A.Z., and 10 by the other, F.N.C. Pressures in the saphenous artery of trained dogs in the standing position were determined by auscultation. Readings were made 5 days a week for from 4 to 8 weeks. Means of individual readings were stable during this time. In the longer series, correlation coefficients were 0.89 for systolic and diastolic pressures, 0.90 for systolic and pulse pressures and 0.61 for diastolic and pulse pres-

tures. The long and short series are summarized as follows:

Pressure	Ns	Ms	S.D.	Mean S.D. <sup>1</sup>
Systolic	57	143	17.8	11.3
	10	121	3.8	7.7
Diastolic	57	70	9.5	8.0
	10	67	2.1	7.4
Pulse	57	64	10.0	7.5
	10	55	3.4	7.8

<sup>1</sup> Mean S.D. of means of individual readings on each dog.

Variation in blood pressure from one individual to another was concluded to be about the same in the dog as in man.

*Brachial peripheral pulse waves related to altitude tolerance and endurance.* T. K. CURETON AND B. H. MASSEY\*. Physical Fitness Research Laboratory, Univ. of Illinois, Urbana.

Several studies indicate that: 1) Endurance performances on the treadmill at 7 m.p.h. at 8.6% grade can be predicted fairly well ( $r = .70$ ) from a combination of systolic amplitude, diastolic surge, diastolic amplitude and angle obliquity. A single test slightly less valid is the area under the pulse wave divided by the surface area of the body ( $r = .60$ ;  $\eta^2 = .70$ ). 2) Significant differentiation is shown between champion swimmers and ordinary competitive swimmers for both men and women. 3) The pulse wave increases with training, especially the area, the systolic amplitude, the diastolic amplitude and the surge. 4) Progressive decline in the pulse wave area and diastolic surge has been shown to parallel artificially induced anoxia in the decompression chamber, progressive fatigue and endurance exercises and the area deteriorates in stasis due to long sitting without appreciable movement. 5) Individual differences are very wide depending primarily upon the state of training of the subject for endurance performances.

At reasonably constant temperature the pulse wave reduces in amplitude and area as subjects are carried to altitude. Preliminary work indicates that the pulse wave is somewhat reduced in area by cold environment and it is amplified with warm conditions, hence the importance of controlled atmospheric conditions for the tests. Static sitting for 1-2 hours will reduce the pulse wave area approximately 30 per cent with a reduction of the area, the systolic amplitude and the diastolic surge characteristic. Fear of the examination also appears to reduce the pulse wave in most subjects. Emotional excitement, especially anticipation of a race, will greatly exaggerate the secondary wave character-

istic and the amplitude of both the primary and secondary waves. The relationship is good between the pulse wave and stroke volume in the quiet state. The pulse wave rating increases with training, is larger in endurance athletes and declines faster after systole and is reduced and slower in older sedentary subjects. The pulse waves are usually larger in the basal, quiet state than in normal sitting or standing.

*Some physiological effects of restricted salt diets in hypertensive and normal rats.* H. G. DANFORD\* AND R. C. HERRIN. Dept. of Physiology, Univ. of Wisconsin Medical School, Madison.

Rats made hypertensive with silk around both kidneys as described by Page were fed control diets containing at least 2% salt mixture and experimental diets containing from 0.3% to no inorganic salts. The systolic pressure was determined by the method of Byrom and Wilson with modifications by Hamilton and Sobin. The rats were observed for at least 30 days with a pressure of 180 mm. Hg prior to the salt restriction. In 8 of the 19 rats on salt restriction, the average systolic pressure decreased 20 to 30 mm., but never remained at a normal of 150 mm. The restricted salt diets favored survival as reported by Grollman and Harrison. Ninety days after salt restriction, 5 of the 12 controls were dead but only 2 of the 19 experimental groups were dead. To ascertain the effect of diets low in salt, normal rats of 40 to 50 gm. were fed a control diet containing 3% salt mixture; *diet 2*, deficient in NaCl, and *diet 3*, deficient in all inorganic salts. The experimental period was 30 days for some rats and 42 days for others. The deficient diets produced a depletion of adrenal ascorbic acid followed by hypertrophy of the gland and a return to its normal ascorbic acid concentration. An involution of the thymus occurred, accompanied in one instance by a lymphopenia.

*Electrolyte concentration of sweat as a differential diagnostic test in hypertension.* DEAN F. DAVIES AND HELEN E. CLARK (introduced by HENRY A. SCHROEDER). Dept. of Internal Medicine and Oscar Johnson Institute, Washington Univ. School of Medicine, and the Barnes Hospital, St. Louis, Mo.

Schroeder has presented clinical evidence for the existence of a group of hypertensives having certain features in common with Cushing's syndrome, which he tentatively has called 'pseudo-Cushing' hypertension. Positive evidence that Schroeder's group represents a distinct entity which differs from other hypertensives both in clinical and laboratory characteristics has been obtained. Conn found high concentrations ( $> 105$  mEq/l.) of sweat sodium in Addison's Disease and low values ( $< 13.5$  mEq/l.) in Cushing's syndrome. A modification of this test has been used in differentiat-

ing Schroeder's hypertensives from normal subjects and from other hypertensives. Subjects were sweated in a saturated steam room for a half-hour at 102° to 110°F. Sweat samples were collected from the back at 15 and 30 minutes and analyzed by the Beckman Flame Photometer for sodium and for chloride by the Wilson-Ball modification of the Volhard method. In the control series the 30-minute sweat sodiums were much lower in summer (mean 32.9 mEq/l.) than in winter (mean 63.6 mEq/l.). No sex difference could be demonstrated among the 21 subjects tested. No significant difference was found between normal women and obese normotensive women. By clinical appraisal of 9 characteristics, 24 women with Schroeder's syndrome were selected. These had a mean 30-minute sweat sodium of 20.4 mEq/l., significantly lower than the lower (summer) normal group (32.9 mEq/l.). Other hypertensives (51.5 mEq/l.), however, were not significantly different from the higher (winter) normal group (63.6 mEq/l.), and therefore had no apparent hyperadrenocortical function.

*Effect of various agents applied to mucosa of secreting stomach on potential difference and rate of secretion of HCl.* THOMAS P. DEGRAFFENRIED II\*, FRED E. COY, JR.\*, F. J. BAJANDAS\* AND WARREN S. REHM. Dept. of Physiology, Univ. of Louisville School of Medicine, Louisville, Ky.

Previously published work (*Am. J. Physiol.* 149: 162, 1947) has shown that there is a relationship between the potential difference across the stomach and the rate of secretion of HCl. It was found that with saline in contact with the mucosa the initiation of secretion was associated with a decrease in P.D., and that the P.D. leveled off in the neighborhood of 40 mv., even though the secretory rate continued to increase. The method used in these experiments and also in the ones reported here was one in which a portion of the stomach was placed in a lucite chamber and arranged so that the blood supply was intact and the P.D. could be measured across the stomach. Histamine, or histamine plus mecholyl, was used to stimulate secretion. It was also shown that ethyl alcohol, or concentrated HCl, applied to the mucosa, or electric current applied across the stomach resulted, once the secretory rate leveled off, in a decrease in both the P.D. and secretory rate. Further experiments were performed with these agents, and also with the following agents applied to the mucosa: NaF, NaCN, NaCNS, procaine, HgCl<sub>2</sub> and ZnSO<sub>4</sub>. It was found that with one exception every time there was a marked reduction in P.D. there was also a marked reduction in secretory rate. In this exception it was found that a portion of the stomach was injured and

the decrease in the measured P.D. was probably due to shunting of the P.D. through the injured part.

*Oxygen dissociation curves of human blood.* D. B. DILL. Medical Div., Army Chemical Center, Md.

The oxygen dissociation curve for the blood of A.V.B. has been described by Dill, Edwards, Florkin and Campbell (*J. Biol. Chem.* 32: 143, 1932). Subsequently this curve was recalculated from a  $p_{HC}$  of 7.1 to a  $p_{HS}$  of 7.4. In addition, dissociation curves were calculated for  $p_{HS}$  values 7.2 and 7.6. The family of three curves was reproduced in a restricted military document, and subsequently by Riley and Cournand (*J. Applied Physiol.* 1: 825, 1949). The purpose of this communication is to point out minor errors in certain parts of the curves corresponding to  $pH$  7.2 and 7.6. The curve showing the dissociation of oxyhemoglobin at  $pH$  7.4 is believed to be correct; it differs slightly from the basic curve at  $p_{HC}$  7.1, this being nearly equivalent to  $p_{HS}$  7.4. However, errors arise when curves at other  $pH$  values are calculated on the assumption that the curves belong to one family: having determined the complete curve at  $pH = 7.4$  and the ratio  $pO_2$  at 50% HbO<sub>2</sub>,  $pH = 7.4$  it has been assumed that  $pO_2$  at 50% HbO<sub>2</sub>,  $pH = a$  the curve at  $pH = a$  can be calculated for all  $pO_2$  values. Recent observations by Roughton (personal communication) prove this assumption incorrect below 10% HbO<sub>2</sub> and above 90% HbO<sub>2</sub>. Hence, until new and direct experimental evidence is obtained, dissociation curves calculated for  $pH$  values 7.2 and 7.6 can be considered valid only between 10% and 90% HbO<sub>2</sub>. Incidentally, Roughton's finding makes invalid within the same ranges dissociation curves recalculated from one temperature to another.

*Determination of natural estrogens in marine eggs by biological and fluorimetric procedures.* J. KENNETH DONAHUE. Dept. of Biology, College of Charleston, Charleston, S. C.

Crude alcoholic-ether extracts of the eggs of 3 species of echinoderms, 2 species of arthropod, and 3 species of teleost fish gave qualitative evidence of estrogenic activity when injected into spayed rats. It was evident that the invertebrate ova had considerably higher potencies than those of the fish ova tested. Highly purified extracts of the eggs of the American lobster and of two species of fish, the alewife and the silver hake, made it possible to carry out rigid bio-assays as well as fluorimetric analyses of their estrogen content. In all cases the biological and fluorimetric results agreed remarkably well. However, whereas the estrogen content of the lobster eggs ranged from 1 to 3  $\mu$ g/m. of eggs (wet weight) that of the fish ova ranged from

only 2 to 30 IU of activity per 100 gm., a negligible quantity.

*Applications of a miniature manometer recording from intracardiac end of a cardiac catheter.* EUGENE J. ELLIS, OTTO H. GAUER, HIRAM E. ESSEX AND EARL H. WOOD. Wright-Patterson Air Force Base, Dayton, Ohio, and Mayo Foundation, Rochester, Minn.

Recording of uniformly reliable intracardiac pressure tracings by means of conventional cardiac catheters is practically impossible because of the pressures generated within the catheter from the accelerations and decelerations of the fluid column associated with the movements of the catheter usually imparted by the heart beat. This difficulty, along with others inherent in recording through a long, narrow-bore thermoplastic tube, can be eliminated by mounting a miniature manometer on the intracardiac end of the catheter. The Gauer-Gienapp manometer has been mounted on a no. 8F cardiac catheter. The manometer, which operates as a differential transformer, has a natural frequency of more than 1000 C.P.S., is linear and temperature-stable. The output of the manometer is amplified by means of a carrier-wave amplifier which can be coupled to suitable recording galvanometers so that the over-all dynamic response is within  $\pm 5\%$  out to 50 C.P.S., with no peaks thereafter. The instrument has been used to study the minimal dynamic response characteristics required for faithful recording of central pulses. The flat portion of the over-all dynamic response curve of the manometer system could be varied at will from 5 out to 50 C.P.S. by switching various electrical shunts into the galvanometer circuit. In dogs systolic, diastolic and mean pressures recorded from the right ventricle, pulmonary artery, left ventricle and aorta were not significantly different, irrespective of whether the sensitivity of the system to sinusoidal pressure variations was uniform out to 5 or out to 50 cycles/sec.

*Induced variations in pulmonary arterial pressure in man.* E. HARVEY ESTES AND ROBERT L. MCWHORTER (introduced by JAMES V. WARREN). Emory Univ. School of Medicine, Atlanta, Ga.

The demonstration of striking alterations in renal function with the application of venous tourniquets to the legs has aroused a great deal of interest in the effects of this procedure on cardiovascular dynamics. For this reason pulmonary arterial pressure was measured in eleven normal subjects before, during and after venous pooling by means of venous tourniquets. The average mean pulmonary arterial pressure fell from 15.3 mm. Hg to 11 mm. Hg during the period of tourniquet application. This is a fall of 28.1% of the average control value. The minimum reduction during the period

of tourniquet application was 9% of the control value. The maximum reduction was 56% of the control value. The reduction in pulmonary arterial pressure during the period of tourniquet application was highly significant statistically. Hamilton has demonstrated that vital capacity is increased in normal recumbent subjects by the application of venous tourniquets. This is due to a reduction in the volume of blood within the pulmonary vascular tree. It is felt that this reduction in pulmonary blood volume is an adequate explanation for the reduction in pulmonary arterial pressure with venous occlusion.

*Mechanism of uneven pulmonary ventilation.* WARD S. FOWLER (introduced by J. H. COMROE, Jr.). Dept. of Physiology and Pharmacology, Univ. of Pennsylvania Graduate School of Medicine, Philadelphia.

Explanations for uneven ventilation have been 1) local stratification of gases and 2) regional variations due to differing percentage volume changes during inspiration. These theories were tested, using continuous analysis of  $N_2$  concentration (Lilly-Hervey  $N_2$  meter) and volume flow of gas expired after one inspiration of  $O_2$ . After  $O_2$  inhalation, alveolar  $N_2$  concentration varied, increasing during expiration, indicating uneven distribution of inspired  $O_2$ . The variation was reduced but not eliminated after 20 seconds inspiratory breath-holding; stratification should have disappeared during breathholding. Mechanisms of regional variation were tested by inspiring a)  $O_2$  alone, b)  $O_2$  followed by air and c)  $O_2$  preceded by air (added  $N_2$  dead space). When inspiration began at the normal expiratory position, changing the sequence of inspired gases did not affect the variation in alveolar  $N_2$  concentration. However, in normal subjects and patients with pulmonary disease, when inspiration began at an extreme expiratory position, added dead space produced greater variation;  $O_2$ -air inhalations in normal subjects gave decreasing  $N_2$  concentrations later in expiration, in patients less variation occurred than with  $O_2$  inhalations. These changes are incompatible with uneven ventilation due solely to differing percentage volume changes, but indicate that certain lung areas fill before and empty after other areas. Areas which fill first will receive more dead space gas than areas filling later, causing uneven ventilation despite equal percentage changes in volume.

*Regeneration of spinal cord of the rat.* L. W. FREEMAN, J. C. FINNERAN\* AND D. M. SCHLEGEL\*. Dept. of Surgery, Indiana Univ. Medical School, Indianapolis.

In a series of over 500 rats which had been subjected to surgical transection of the spinal cord under direct observation, functional recovery has been observed in a fairly high percentage of those surviving beyond two

months. Mortality in the entire series has been extremely high during early days after operation, and the many factors contributing to this are analyzed. They include anesthesia, hemorrhage, temperature variations, rupture of the bladder, trauma with subsequent self-annihilation, inadequate fluids, intercurrent infections, urinary tract calcinosis and others. A rational outline of care of these animals is presented, including careful periodic expression of the bladder, adequate fluids and special attention to diet and environment. The histological picture in animals showing functional regeneration reveals the down growth of both spinal nerve root components and components presumed to be internuncial. It is argued that positive experimentation of this type adds force to the re-opening of the question of functional regeneration in the central nervous system, patterned after the re-opening of many such discussions which have followed recent advances in neurological investigations. Failure of regeneration is analyzed in terms of local and general factors, with species differences being described in terms of general conditions.

*Response of acoustic system in clicks.* ROBERT GALAMBOS *et al.* Psycho-Acoustic Lab., Harvard Univ., Cambridge, Mass.

Various nuclei of the acoustic system of the cats have been explored with microelectrodes. Each ear is represented bilaterally in the nervous system from the level of the cochlear nucleus to the cortex. The latency of the response evoked by the click and its duration increase as the acoustic tract is ascended. Within a given nucleus the post-synaptic activity aroused varies in latency, and may attain unexpectedly large values (e.g. 50 msec. at the cochlear nucleus). These studies demonstrate the very considerable complexity of the electrical response to a brief (1.5 msec.) acoustic pulse.

*Retention of cardiac, salivary and motor conditional reflexes.* W. HORSLEY GANTT AND URSULA TRAUGOTT. Johns Hopkins Univ. School of Medicine, Baltimore, Md.

Upon the function of retention depends the ability of an organism to react to experimentation. Previous studies have measured only the motor (somatic muscular) response and have paid too little attention to the emotional and visceral retention. On the other hand the data of clinical psychiatry, while supporting evidence of long retention, do not control the factor of intermediate reinforcement of the original situation. In several laboratory dogs stable conditional reflexes (cr) were formed to food or to pain (faradic shock). In addition to the specific salivary and motor components,

the more general visceral components representing the emotional response (heart rate, respiration) were recorded in both normal and pathological dogs. There was no repetition of the original conditional stimulus (cs) until the animal was brought in for a retention test; criterion for retention—whether the individual gave the cr on the very first presentation of the cs. In one dog there was perfect retention of the motor cr to faradic shock after an interval of 8 months, 2 years, and 4 years, with loss of differentiation after 4 years. In another dog, extremely phlegmatic, after 16 months there was no retention of the motor (cr pain), but some retention of the cardiac component and marked retention of the general emotional pattern. In Peik with food crs the evidence for retention of cardiac components was made even stronger by attempting to extinguish the cr (repetition cs without food). Although the salivary component disappeared in 1 or 2 days and did not reappear in 1 month and 1 year, respectively, the cardiac cr could not be extinguished and was even greater after 1 year. Pathological animals, as well as patients, show a persistent retention without reinforcement—7 or 8 years with one dog for cardiac, sexual and other visceral responses to situations of conflict. Conclusion: emotional components of a cr (respiratory and cardiac) are retained much longer than the specific motor or secretory; retention is even longer in pathological animals than in normal.

*Cardiac arrhythmias* (Moving picture). P. C. GAZES, R. P. WALTON, O. J. BRODIE AND J. S. LEARY (introduced by T. G. BERNTHAL). Dept. of Pharmacology, Medical College of South Carolina, Charleston.

This film is unique in presenting direct views of the heart with simultaneous views of the ECG tracings as they are being recorded. A variety of arrhythmias are produced by anoxia, by electrical stimulation, by mechanical stimulation, by pilocarpine, by epinephrine and by digitalis. With anoxia the heart rate drops to about 25 beats per minute and is still of a sinus node mechanism. Apparently nodal rhythm or some idioventricular rhythm did not develop because all the conducting tissue is proportionally depressed. A U wave also developed, which disappeared with respiratory improvement. Auricular and ventricular fibrillation are illustrated.

Pilocarpine, a typical cholinergic drug, produced marked sinus bradycardia and then an idioventricular rhythm (rate 22). This shows the intense block produced at the sinus node, thus allowing a lower center to take over, whereas with anoxia a sinus mechanism persisted at this slow rate. With the vagi intact, epinephrine produced a distinct sinus bradycardia with vagal arrhythmia. After the vagi were sectioned, epinephrine

increased the rate and a run of ventricular tachycardia occurred. The fact is visually demonstrated that the heart, without the protection of the intact vagi, may be thrown at a stage near ventricular fibrillation, whereas the same heart is subject to a conspicuously lesser degree of danger when the protective vagal reflexes are intact. Possible hazards involved in the clinical administration of sympathomimetic compounds following atropine are suggested by this demonstration. Digitalis produced prolongation of the PR interval, coupled ventricular extra systoles, ventricular tachycardia, and final ventricular fibrillation. A run of bi-directional ventricular tachycardia was seen. In viewing the heart and ECG together it can be seen that the downward deflected QRS complex is due to an impulse from the right ventricle and the upward QRS from the left ventricle. This supports Marvin's theory that in bi-directional ventricular tachycardia there are two separate ectopic foci, one from each ventricle.

*Uptake of radioactive phosphate by red blood cells\**

CHALMERS L. GEMMILL. Dept. of Pharmacology, Univ. of Virginia Medical School, Charlottesville.

In a previous communication Pertzoff and Gemmill (*J. Pharmacol. & Exper. Therap.* 95: 106, 1949) described various factors influencing the rate of uptake of radioactive phosphorus in the form of phosphate by human red blood cells. In these experiments, it was noted that there was considerable variation in the rate of uptake not only in blood of different donors but also from the same donor. However, in the same experiment, using different samples from the same blood, good agreement was obtained. It was observed that sodium barbital and ether retarded the uptake of radioactive phosphate ions by the red blood cell but methadon and urethane had no effect on this process. These experiments have been extended by modifying the former technique and by studying the effects of glucose and insulin on this process. A few preliminary experiments have shown no marked changes in the velocity of uptake with varying concentrations of glucose or by the addition of insulin.

*The functioning isolated rat spinal cord.* R. W. GERARD,

R. D. TSCHIRGI, H. JENERICK AND J. Z. HEARON.  
Dept. of Physiology, Univ. of Illinois, Chicago.

This paper extends results reported previously concerning functional and metabolic correlations in the isolated, perfused spinal cord of the rat. Since those earlier experiments our preparation has failed to function in many instances and results have been erratic. Use of Knox gelatin (4% solution) instead of egg albumin in the Tyrode's solution has increased the number of successful preparations. The operative procedure has been modified to include the lumbar cord segment with

its longer more accessible roots. Average glucose utilization, 7.4 mg/gm. wet weight/hr. (range 7.1-8.0), gives a calculated  $QO_2$  of 5000. Observed  $QO_2$  (van Slyke determinations) averaged 4250 (range 3900-4450) so that, as often reported for *in vivo* experiments, more glucose disappears than could be oxidized. The R.Q. averaged 0.99 (range 0.97-0.99). Although succinate is unable to restore or maintain synaptic conduction, following a 15-minute period of aglycemia, it nevertheless disappears from the perfusate in large quantities (57 mg/gm. wet weight/hr.). A simple energetic explanation of its inactivity is further unlikely since succinate added to just insufficient glucose will not maintain function. In light of our previous findings, this suggests that the alpha-ketoglutarate to succinate step may represent a metabolic *sine qua non* for mono-synaptic spinal reflexes. Initial experiments indicate that low concentrations of sodium pentobarbital (1-3 mg.%) halve the threshold and increase number of active units, whereas anesthetic levels (4-6 mg.%) profoundly or fully suppress reflex response and increase threshold some 50%.

*Factors concerned in the tension development of a summated response of skeletal muscle.* ARTHUR S. GILSON, JR. Dept. of Physiology, Washington Univ. School of Medicine, St. Louis, Mo.

Experiments were undertaken to investigate quantitatively the thesis that the development, maintenance and relaxation of tension in the isometric tetanus of skeletal muscle may be due merely to the mechanical summation of rapidly succeeding twitch responses. Muscles used were the long neck retractor of the turtle and the gastrocnemius of the frog, excised, suspended in moist air and stimulated directly by supramaximal condenser shocks. Progressive development of fatigue caused diminution of tension development and slowing of the twitch responses. A second shock following a first by an interval certainly not longer than 1.5 msec. and perhaps less (for frog gastrocnemius) results in a response giving greater tension development than that of the single twitch response. The tension added as the result of such a second shock is least with short shock intervals, and increases toward the tension developed by the single twitch as the shock interval is increased. The tension contributed by the second response also depends upon the residual tension from a first response and, with moderately long shock intervals, may be nearly equal to the tension developed by a single twitch starting from corresponding rest tension. Slowing of the individual component responses occurs and does give some smoothing of a partial tetanus. Such slowing does not seem adequate to account for the maintained tension of certain smooth tetanic responses having

marked persistence of contraction after cessation of stimulation. It seems possible that in such responses there may develop a true steady-state condition of the contractile mechanism.

*Measurement of gastric mucosal damage after alcohol in the rat.* RHODA GRANT AND A. C. IVY. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

The damage produced in the glandular part of the rat's stomach by alcohol was studied in the interests of finding an assay method for agents which increase the resistance or promote healing of the mucosa. Damage, graded in arbitrary units, at 2, 23, or 40 hours after 50% or 35% to 40% ethyl alcohol in volumes of 1 cc. and 2 cc/100 gm. rat, given by stomach tube, was statistically compared. The damage by 1 cc. and by 2 cc. differed significantly, as did that between 2 hours and 23 to 40 hours. For the number of rats used the difference between 1 cc. 50% and 1 cc. 35% to 40% was not significant. Microscopic examination, by showing the depth of erosions, supported the macroscopic grading, and showed that the healing occurring between 2 and 23 to 40 hours was due in part to epithelization. Daily injections for 6 to 13 days of an anti-ulcer preparation of urine (Ayerst, McKenna and Harrison) raised the mucosal resistance judging by the significant difference at 2 hours between preparation-treated and saline control groups. The method is not suitable for quantitative measurement of microscopic changes; therefore, it was impossible to evaluate quantitatively the effect of the preparation on epithelization occurring between 2 and 23 to 40 hours. The reproducibility of the test, seen in 17 different control groups (92 rats) over 11 months, suggests that the method may prove useful in testing agents which influence the resistance or healing of the glandular part of the stomach.

*Regulation of sodium excretion.* D. M. GREEN AND A. FARAH. Univ. of Washington, Seattle.

Normal dogs respond to sodium loading by an increase in urinary sodium excretion. The increase is due primarily to a reduction in tubular reabsorption of sodium (GREEN, D. M. AND A. FARAH. *Am. J. Physiol.* In press). The present studies were designed to determine the mechanism by which these changes are regulated. It was found that hypophysectomized or adrenalectomized dogs responded to sodium loading by increases in sodium excretion comparable to those displayed by control animals. They differed in the amount of water excreted relative to sodium. Hypophysectomized animals increased urinary sodium concentration slowly to maximum values approximately two-thirds of the plasma sodium level. Adrenalectomized animals promptly increased urinary sodium concentration to

values which equalled or exceeded the plasma sodium level. The failure of hypophysectomy or adrenalectomy to abolish the response to sodium loading suggested the possibility of local renal regulation of sodium excretion. Injection of sodium chloride solutions directly into one renal artery promptly increased the sodium excretion of the injected kidney. The total sodium output greatly exceeded that displayed by the contralateral kidney. The rise in excretion was associated with a marked reduction in blood flow during injection.

*Comparison of volume of coronary blood flow simultaneously determined with nitrous oxide method and rotameter.* P. A. GREEN\*, DONALD E. GREGG AND L. J. CZERWONKA\*. Medical Dept., Field Research Laboratory, Fort Knox, Ky.

A comparison has been made of the simultaneous values for left coronary artery blood flow in the open-chest, anesthetized dog as measured indirectly by the nitrous oxide method, and directly by an optically recording rotameter. The inflow side of the rotameter was connected to a carotid artery, the outflow connection was tied within the left coronary ostium. Nitrous oxide saturation and desaturation curves were established by blood samples drawn from the rotameter and from a catheter within the coronary sinus. Based on dyed heart weight, the nitrous oxide values differed maximally from the rotameter measurements by +50 to -17% in 15 comparisons in 10 dogs. In 11 comparisons in 9 dogs, a somewhat better correlation (+18 to -10%) was found between the two methods when the rotameter flow measurements were based on left heart weight (left ventricle, left atrium and total septum). The nitrous oxide values exceeded the rotameter flow values in 10 of 15 and 9 of 11 comparisons when based on dyed and weighed myocardium, respectively. Since tests have established that the maximum error with the rotameter approximates 5%, these sizeable differences between the values obtained with the two methods could arise either from the inaccuracy of the nitrous oxide method as applied to the anesthetized dog, or from inability to determine accurately the weight of the myocardium fed by the left coronary artery.

*Abolition of gametokinetic response in Rana pipiens with adrenolytic substance benzodioxan and possibility of a test for diagnosis of pheochromocytoma.* R. B. GREENBLATT, SARAH CLARK AND R. M. WEST (introduced by W. F. HAMILTON). Dept. of Endocrinology, Univ. of Georgia School of Medicine, Augusta.

Injection of piperidylmethyl benzodioxan (Merck) into the male frog (*Rana pipiens*) in amounts varying from 0.5 mg. to 2.5 mg. partially or completely blocked the gametokinetic response to human pregnancy urine, epinephrine, ICSH and chorionic gonadotrophin. Com-

plete section of the spinal cord failed to alter the frog's gametokinetic response to chorionic gonadotrophin or its inhibition by benzodioxan. Benzodioxan did not inhibit the production of ovarian hyperemia by ICSH and human pregnancy urine in the immature female Sprague-Dawley rat. The possible role of the pituitary as an adrenergic link in the gametokinetic response has not been established. The trigger mechanism in the gametokinetic response may be the stimulation of some neuromuscular ejaculatory apparatus or the activation of an epinephrine receptor substance. A possible diagnostic test for pheochromocytoma has been proposed, the criterion being the production of gametokinesis in the male frog upon the injection of serum from the patient suspected of having a pheochromocytoma.

*Quantitative aspects of inhibition of gastric secretion by pyrogens.* M. I. GROSSMAN AND DELBERT BLICKENSTAFF. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

Pyrexia was induced by the intravenous injection of pyrogen, and its effect upon the rate of gastric acid secretion induced in gastric pouch dogs by injection of histamine every 10 minutes was observed. The highest dose of bacterial pyrogen used (10  $\mu$ g/kg.), produced an average temperature rise of 3.0°F., and an average of 72% inhibition of secretion for the 3-hour period following the injection. For both pyrogens used the relation between log dose of pyrogen and pyrexia or inhibition is linear over a large portion of the range of doses studied. In no individual test did gastric secretory inhibition occur without pyrexia. There was a lag of about one hour between the onset of pyrexia and the onset of inhibition; there was a similar lag between the subsidence of pyrexia and the return of secretion to control levels. Because of these lags, early in the course of a test pyrexia often occurred without secretory inhibition, whereas late in the test secretory inhibition without pyrexia often was seen. Pyrexia, therefore, is considered not to be directly responsible for the depression of secretory activity.

*Effect of a 20° head-up tilt on cerebral blood flow in essential hypertension.* JOSEPH H. HAFKENSCHIEL, CHARLES W. CRUMPTON, JOHN H. MOYER, HENRY A. SHENKIN AND WILLIAM A. JEFFERS. Robinette Foundation, Medical Clinic, Hospital of the Univ. of Pennsylvania, and Harrison Dept. of Surgical Research and Dept. of Pharmacology, Univ. of Pennsylvania School of Medicine, Philadelphia.

Cerebral blood flow and oxygen consumption were measured by the nitrous oxide method in eight patients before and after 20 minutes of tilt in a study of the effects of semi-erect posture on cerebral hemodynamics and oxygen metabolism in hypertension. Seven other

patients were studied similarly 2 weeks to 21 months after sympathectomy. The tilting responses of each group are compared with the results obtained using the same procedure in 5 normotensive patients studied by Shenkin (*Am. J. of Med. Sc.* 216: 713, 1948). The mean carotid pressure was reduced by the tilt in all three groups, this being the only significant change in the sympathectomy group. Cerebral blood flow and cerebral vascular resistance were reduced in the unoperated hypertensives. Cerebral oxygen uptake was unchanged while oxygen content of jugular venous blood dropped from 10.8 to 9.3 vol.% in this group. These findings suggest that tilting hypertensive patients reduced the cerebral blood flow. The increased cerebral vascular resistance was reduced by the tilt but not to the extent necessary to compensate for the lowered cerebral arterial pressure. Oxygen consumption was maintained at the cost of a lowered cerebral oxygen tension. The tilt response of hypertensives after sympathectomy more closely resembled that of normotensives.

*Growth and body structure of the albino rat on diets in which one of the major foodstuffs predominates.* JOHN HALDI AND WINTREY WYNN\*. Dept. of Physiology, Emory Univ. School of Dentistry, Emory University, Ga.

A study has been under way in our laboratories for over two years to determine whether a large amount of sugar in the diet over a long period of time produces any deleterious effects on the animal organism. Evaluation of the effect of the large sugar intake is made by comparative observations on animals maintained on a high fat and a high protein diet. These observations are to be extended over two generations of albino rats deriving approximately 60% of their total caloric intake from either carbohydrate (sucrose), protein or fat. Data are to be obtained on the number of litters born, the number of offspring, weight of offspring at birth, viability of offspring, growth curves, longevity, radiographic and histological structure of the bones in the active growing stage, weight of the individual organs, histological structure of the internal organs, water, protein, fat, total ash, calcium and phosphorus content of the eviscerated body, pyruvic acid, lactic acid and hemoglobin content and the red and white cell count of the blood. The teeth are to be examined for any evidence of caries and for any abnormalities that might occur. This present preliminary report is concerned with only a few limited phases of the problem, viz: weight curves, fat, protein, calcium, phosphorus and total ash of the bodies, weight and histological structure of the organs of the animals of the first generation. From the data now available and to be presented, it appears that a large amount of sugar in the diet has no disadvantage as regards the growth and structure of the organism.

*Absence of an acute pressor response to desoxycorticosterone in hypertensive dog.* CHARLES E. HALL. Carter Laboratory of Physiology, Univ. of Texas, Medical Branch, Galveston.

The hypertensive effect of desoxycorticosterone acetate has been demonstrated in rats, dogs and man. In 1948 Goldman and Schroeder reported that the intravenous administration of 5 mg. DCA in propylene glycol caused an acute pressor reaction maximal within 30 minutes in hypertensive patients, but not in normotensives. Since it is held by some investigators that essential hypertension is of renal origin, we decided to test the response to DCA of dogs with renal hypertension. The hormone was administered intravenously to 3 hypertensive dogs and 2 normotensive controls in doses of from 4 to 12 mg. in 2 to 2.5 cc. propylene glycol. The solvent was used as a control injection on other occasions. Six trials with DCA and 6 with propylene glycol were made on 2 of the hypertensive and both control dogs. Four trials with DCA were made on the remaining hypertensive dog. Blood pressure recordings from the femoral artery, using a strain gauge pressure transmitter and an oscillograph, were made at 0, 15, and 30 minutes, and occasionally, some hours after injection. A constant pressor response to DCA was not observed. The few elevations of blood pressure which were observed occurred in both hypertensive and normotensive dogs and followed the injection of solvent alone as frequently as with DCA. More commonly there was no change in blood pressure. No difference between the lower and higher doses of DCA was observed. It is concluded that desoxycorticosterone acetate does not elicit an acute pressor response in dogs with renal hypertension.

*Histamine content of canine pancreatic tissue and pancreatic juice.* G. A. HALLENBECK, M. DWORETZKY AND C. F. CODE. Section on Physiology, Mayo Clinic and Mayo Foundation, Rochester, Minn.

Pancreatic juice was obtained from 3 trained dogs by the method of Thomas. The histamine equivalent of pancreatic juice was estimated by a modification of procedures described by Barsoum and Gaddum and by Code. The histamine equivalent of pancreatic tissue was determined by this method after extraction of fresh pancreas with 10% trichloroacetic acid. Histamine was undetectable (less than 2.5  $\mu\text{g/l.}$ ) in pancreatic juice secreted in response to 1) intraduodenal instillation of 20 ml. of N/10 HCl every 15 minutes and 2) intravenous injection of one unit/kg. body weight of Lilly's secretin every 10 minutes, and 3) subcutaneous injection of 0.5 mg. of pilocarpine every 10 minutes. Among 8 samples of juice obtained in response to a meal of raw horse-meat, histamine was undetectable in 5, present as a trace (2.5-5.0  $\mu\text{g/l.}$ ) in 2, and present in a concentration of 7.5  $\mu\text{g/l.}$  in one instance. Among 5 samples of juice

secreted in response to 1 mg. of histamine given subcutaneously a histamine-like substance was present in concentrations ranging from 6.3 to 8.4  $\mu\text{g/l.}$  in 4 samples and occurred as a trace in one sample. Thus except in instances in which histamine was given subcutaneously, the presence of a histamine-like substance in pancreatic juice was unusual. This is a notable contrast to the usual presence of a histamine-like substance in gastric juice secreted in response to a variety of stimuli. Canine pancreatic tissue regularly contained a histamine-like substance in quantities which could be readily assayed. Values among 5 dogs ranged from 3.1 to 13.4  $\mu\text{g/kg.}$  of fresh pancreas.

*Some indications of cyclic reproductive activity in the non-ovulating rabbit.* CLARA HAMILTON. Dept. of Biology, Univ. of Georgia, Athens.

An investigation is being made to determine if there are cyclic reproductive changes in non-ovulating rabbit, since, in this organism, there is always a continuous series of ripening and degenerating follicles, and there are always present follicles capable of maturing and rupturing within 10 hours after an adequate stimulus. Studies of the vaginal smear, histology of the reproductive tract and blood estrogen levels are being made. With the aid of a glass tube inserted through the vestibule into the vaginal orifice, smears of the vaginal contents are made and stained according to the Shorr procedure. Daily smears have been made on 6 rabbits for over 100 days each. Study of these smears shows cyclic recurrence of peaks of relative abundance of certain distinct cell types at 4- to 6-day intervals. A histologic examination of thin sections of the reproductive tract, also stained by the differential Shorr stain, indicates slight alterations correlating with the changing vaginal smear and locates the source of certain of these cells. To study blood estrogen levels, ovariectomized guinea pigs, primed several days previously with estradiol benzoate (Progynon B; courtesy Schering Corp.), are given 0.05 cc. whole rabbit blood perivaginally (Hartman-Littrell method; *Endocrinology* 49: 292); opening of the vaginal closure plate is a positive response. This latter portion of the study has only begun, but indications are that at the time cornified cells are relatively most abundant there is a higher blood estrogen level.

*Influence of temperature on pressure-volume.* J. HENRY, H. JACOBS, A. KARSTENS AND O. GAUER. Aero Medical Laboratory, Wright-Patterson Air Force Base, Dayton, Ohio.

It is of value to know the manner in which changes in the tone of the venous system of the limbs affect the volume of blood contained by them at any particular pressure. A pressure plethysmograph was made

in which a man could stand immersed up to the groin. By bending forward, mean venous pressure in the legs was reduced to approximately 40 cm. water. The volumes of the veins at pressures intermediate between zero and 40 cm. water were determined by applying various degrees of fluid counter-pressure; and those in the range of 40–120 cm. water by increasing venous pressure by pressure breathing. A change from sweating with vasodilation to shivering was obtained by adjusting bath temperatures variously in the range from 44° C. to 18° C. A cuff technique, whereby blood was trapped in the legs, was employed to confirm the data obtained by the pressure plethysmograph. Similar results were obtained in the 4 subjects studied. In a typical case at all temperatures, the volume increased rapidly and by the same amount, with rises in pressure up to 30 cm. water. The final volume at this pressure was 400 cc. At pressures from 40 to 120 cm. water, the elastic limits of the system played an increasing part, and volume increments for increased pressure decreased correspondingly. This elastic limit was attained more rapidly in the cold limbs. In one case, the final volume pooled at 18° C. and 120 cm. water pressure was 570 cc., but at 44°C. and the same pressure, the volume was 840 cc. The results show that at pressures in excess of 30 cm. water, the capacity of the venous system in the legs is significantly increased by vasodilation and decreased by chilling.

*Principles underlying behavior of body components under rapidly applied forces.* CARL M. HERGET. Medical Div., Army Chemical Center, Md.

Body components suffer distortion when a force is applied to them. This distortion is propagated along the component with a velocity which has a definite value in any given case. The speed of travel determines how much strain is built up in the region of impact before neighboring regions begin to share in the motion and tend to relieve the strain. Whether or not the component suffers injury thus depends in part upon its own elastic behavior (i.e. upon the speed with which this disturbance travels) and upon the magnitude and duration of the applied force. Examples are given showing the propagation of a distortion along the abdominal fascia of the dog, and the behavior of dog's ribs, under a force of several milliseconds duration which attained a peak value of 400 to 500 g. It is concluded that many individual body components can withstand forces of hundreds or even thousands of g for a short time.

*Effect of sudden changes of inspired air  $pO_2$  on alveolar air gas tensions.* FRED A. HITCHCOCK AND RALPH W. STACY. Laboratory of Aviation Physiology and Medicine, Ohio State Univ., Columbus.

A study of the effects of sudden changes in the  $pO_2$  of inspired air on the  $CO_2$  and  $O_2$  tension of alveolar air has been carried out by means of the mass spectrograph developed in this laboratory. The reduction of  $pO_2$  of inspired air was produced, first, by breathing gas mixtures with low oxygen content and, second, by explosive decompression. In all experiments the rate and depth of respiration was kept constant. When low oxygen tensions were breathed, the  $pO_2$  of alveolar air reached its low level at the end of the 8th to the 10th respiratory cycle. Each breath reduced the  $pO_2$  of the alveolar air by about one third of the difference between the value at the end of the preceding breath and the terminal value. Following explosive decompression the  $pO_2$  reached its low level in a shorter time than the response of the instrument. The new equilibrium was usually reached before the involuntary expulsion of air which resulted from explosive decompression was complete. In both sets of experiments the  $pO_2$  of alveolar air bore a straight line relationship to the  $pO_2$  of inspired air, the difference between these two being approximately 30 mm. Reduction of the  $pO_2$  of the inspired air causes first a rise, then a fall of the alveolar  $pCO_2$ . These results may be tentatively explained on the basis of shifts in the carbon dioxide dissociation curve.

*A comparison of cardiac output obtained by the direct Fick and pressure pulse contour methods in the open-chest dog.* R. A. HUGGINS, E. L. SMITH AND M. A. SINCLAIR. Dept. of Pharmacology, Baylor Univ. College of Medicine, and Dept. of Physiology, Univ. of Texas Dental School, Houston.

Barbital Na, 300 mg/kg. was used as the anesthetic. The chest was opened and a large rotameter inserted on the venous side so that the total venous return could be measured. These data are not reported. For the direct Fick determination  $O_2$  consumption was measured by connecting the dog to a McKesson Recording Metabolism machine and blood  $O_2$  by the micro-method of Roughton and Scholander (*J. Biol. Chem.* 148: 541, 1943). Cardiac output was also calculated from central pulse contours (HAMILTON AND REMINGTON, *Am. J. Physiol.* 148: 14, 1947). Twenty-three simultaneous cardiac output determinations were made on 5 dogs. The average difference between the results with the 2 methods was  $\pm 12.5\%$  with a range from  $-13.7$  to  $+59\%$  and a coefficient of correlation of  $r = +0.985$ .

*On tertiary peristalsis of esophagus of the cat.* KAO HWANG (introduced by A. C. IVY). Univ. of Illinois College of Medicine, Chicago.

Tertiary peristalsis (propulsive movements of the denervated lower portion of the esophagus) has been

studied in 11 cats after complete vagotomy by the technique used by Jurica (*Am. J. Physiol.* 77: 371, 1926) except that only local anesthesia was used. These animals were examined with barium meal under fluoroscopy without anesthesia usually within one hour after the operation. At first only the cervical portion of the esophagus was active and the lower two thirds was paralyzed and dilated. After a period of quiescence of from less than one hour to 2 to 3 days (55% in 3 hours) tertiary peristalsis appeared over the lower third of the esophagus, which persisted and served to evacuate contents into the stomach without dilation during periods of observation up to 5 months. During the initial period of quiescence the paralyzed lower third of the esophagus showed peristaltic activity in response to mechanical stimulation and to parasympathomimetic drugs. Carbaminoylecholine gave more consistent effects than urecholine and prostigmine. Fully developed tertiary peristalsis and peristalsis of the normal esophagus were neither stimulated nor inhibited by these drugs or epinephrine but were paralyzed for a short period followed by paresis after dibutyltoline, atropine and tetraethylammonium chloride. The denervated esophagus seems more susceptible to the muscarinic blocking agents. The cardia in most cats showed persistent atonia after the vagotomy. The theory that cardiospasm develops as a result of vagotomy is not supported by the present work.

*Effect of calcium, citrate and phosphate ions on autonomic ganglia.* ALEXANDER G. KARCZMAR\* AND THEODORE KOPPANYI. Dept. of Pharmacology, Georgetown Univ. School of Medicine, Washington, D. C.

Magnitude of vasopressor effects of intravenously injected acetylcholine and nicotine was used as measurement of sympathetic ganglionic activity in fully atropinized dogs. Calcium chloride up to 450 mg/kg. did not affect these responses appreciably. Sodium citrate in moderate doses (about 100 mg/kg.) occasionally potentiated acetylcholine and nicotine pressor effects, and in larger doses (up to 1.0 gm/kg. in divided doses) greatly diminished or abolished such pressor effects. Sodium hexametaphosphate (up to 300 mg/kg. in divided doses) and other organic phosphates also abolished acetylcholine and nicotine pressor effects. Phosphate compounds and especially the citrate produce a fall of blood pressure, reversible cardiac damage, an initial hyperpnea and subsequently a prolonged apnea. Vasodepressor response and cardiac phenomena were inhibited by calcium chloride but not by atropine. Whenever sympathetic ganglionic activity was depressed by either citrate or phosphate, calcium produced some return of acetylcholine and nicotine pressor effects. When ganglia were depressed by massive

doses of tetraethyl pyrophosphate (TEPP) (*Science* 106: 493, 1947) then calcium produced only a slight return of ganglionic responsiveness. Citrate (100-300 mg/kg.) administered after calcium produced, however, a pressor effect of its own, and subsequently restored full acetylcholine, but not nicotine pressor effects. Whether the citrate reversal of ganglionic paralysis in respect to acetylcholine but not to nicotine can be explained in terms of Bronk's findings of its direct stimulating effect on ganglia remains to be determined. It seems to point, however, to the existence of a non-cholinergic factor in ganglionic excitation (*Federation Proc.* 8: 309, 1949).

*Further studies on cinchophen peptic ulceration.* K. S. KIM\*, D. F. MAGEE\* AND A. C. IVY. Univ. of Illinois College of Medicine, Chicago.

One fraction of gastric mucin contains glucuronic acid. Orally administered mucin is known to afford partial protection against peptic ulceration in Mann-Williamson dogs and in dogs to which cinchophen has been administered. It has been suggested that conjugation of toxic substances with glucuronic acid may result in a decrease of available glucuronic acid for mucin synthesis with a consequent loss of an important protector of the gastric mucous membrane. In this study we undertook to investigate the effect of cinchophen upon the urinary glucuronic acid and upon the pyloric secretion in dogs, which is mainly mucus. Oral administration of cinchophen in doses of 70-150 mg/kg. in dogs and of 200-400 mg/kg. in rabbits increased the urinary glucuronic acid output. As rabbits do not develop cinchophen peptic ulceration, this observation throws some doubt upon the glucuronic acid depletion theory. In chronic pyloric pouch dogs, administration of 150-200 mg/kg. of cinchophen invariably reduced, within 3 days of its commencement, the 4-hourly output of juice by 75%, often by more. This decrease may be an important factor in the etiology of cinchophen ulcer.

*Knowlesi malaria in monkeys. II. A first step in separation of mechanical pathologic circulatory factors of one sludge disease from possible specific toxic factors of that disease.* MELVIN H. KNISELY, WARREN K. STRATMAN-THOMAS, THEODORE S. ELIOT AND EDWARD H. BLOCH. Division of Anatomy and Preventive Medicine, Univ. of Tennessee Medical School, Memphis; Hull Laboratory of Anatomy, Univ. of Chicago; and Dept. of Anatomy, Medical College of the State of South Carolina, Charleston.

Kniseley, Bloch, Eliot and Warner (*Science* 100: 431, 1947) reported circulating sludged blood in human patients diagnosed as having 50 human diseases. The

main experimental attack aimed at understanding how sludge damages the body has been carried out using rhesus monkeys infected with knowlesi malaria (*J. Nat. Malaria Soc.* 4: 285, 1945) and the motion picture described therein. During Stage II of that disease each parasitized red cell is coated with a sticky opsonizing material which permits (and is necessary for) spleen and liver phagocytes to ingest the parasitized erythrocyte. Usually during some parasite segmentation period when the parasite count rises from 5 to 30% of the monkey's red cells, a second semi-rigid precipitate suddenly forms between and around all blood cells changing all blood to a thick, pasty Stage III circulating sludge. This sludge forcibly resists passage through arterioles and capillaries; flow rates sharply decrease; stagnant anoxia of endothelium permits rapid plasma loss through venule walls, decreased venous return and death. Forty monkeys died thus; many with but 50 to 200 parasites per 100 erythrocytes and the Stage III sludge. All died within 12 hours; most within 6 hours. High heparin dosage prevented formation of opsonins and sludge; permitted development of high parasite counts in fluid, rapidly flowing, blood. Animals with from 1000 to 1800 parasites per 1000 erythrocytes lived an additional 12 hours or more after the parasite count was above 500, until parasites consumed all hemoglobin. Stage III sludge kills quicker than possible but undemonstrated parasite toxins.

*Laboratory demonstration of methods currently used for microscopic studies of circulation in living internal organs of animals, and sludged blood in human beings.* MELVIN H. KNISELY, LOUISE WARNER AND LEROY FINNEY. National Naval Medical Center, Naval Medical Research Institute, Bethesda, Md., and Dept. of Anatomy, Medical College of the State of South Carolina, Charleston.

These methods have been described in the literature. KNISELY, BLOCH, ELIOT AND WARNER, *Science* 100: 431, 1947; M. H. KNISELY, in E. V. COWDRY, *Microscopic Technique in Biology and Medicine* (2nd ed.). Baltimore: Williams and Wilkins, 1948. KNISELY, BLOCH, ELIOT AND WARNER in O. GLASSER, *Medical Physics* (2nd ed.). Chicago: The Yearbook Publishers. In press.

*Relation of height and age to reflex time.* G. CLINTON KNOWLTON AND LOUIS P. BRITT\*. Dept. of Physical Medicine, Emory Univ. School of Medicine, Atlanta, Ga.

Reflex time and standing height was measured on 700 individuals ranging from one month to 42 years of age. This sample was screened to exclude all individuals having any history of neuromuscular disorder. Reflex time was taken as the period from mechanical stimu-

lation at the patella tendon to the first appearance of the action potential at the motor point of the rectus femoris muscle. A group consisting of males 6 to 20 and of females 7 to 20 years of age appears homogeneous in that for them the reflex time of the knee jerk can be predicted from body height without regard to age. The formula, time (in msec.) = 0.093 height (in cm.) + 1.4 is the regression line of time on height derived from the correlation coefficient. The probable error of estimate is 0.6 msec. This restricted sample consists of 1192 reflexes from 596 individuals. The number of observations on individuals outside this age group is too small to give valid prediction formulae, but is adequate to show that these excluded age groups do not fit the formula given above. Before the 6th and after the 20th year the reflex time is longer in proportion to height than it is for the intermediate age group. Whether this represents a change that occurs in a given individual in passing these ages can only be determined from repeated observations on the same individuals over a span of years.

*Eserine-like activity of adenylic acid and of water-soluble adenosine-triphosphate (ATP).* THEODORE KOPPANYI AND ALEXANDER G. KARCZMAR\*. Dept. of Pharmacology, Georgetown Univ. School of Medicine, Washington, D. C.

Inorganic and organic phosphates were tested in anesthetized dogs as to their ability to potentiate muscarinic and nicotinic effects of acetylcholine and responses to preganglionic nerve stimulation. Adenylic acid and ATP showed more marked effects than other phosphates with exception of DFP-type anticholinesterases. Adenylic acid and ATP produced vasodepressor effects on intravenous administration not inhibited by atropinization. Adenylic acid (0.5-6.0 mg/kg.) potentiated the vasodilator effects of acetylcholine and cardiac inhibition following stimulation of peripheral end of divided vagus. ATP (0.1-10.0 mg/kg.) produced, somewhat less consistently, similar effects. In atropinized animals adenylic acid potentiates the pressor effects of acetylcholine and nicotine as well as hemodynamic responses following faradic stimulation of the cephalic end of the divided vago-sympathetic. ATP potentiates the latter, but affects negligibly the response to acetylcholine and nicotine. Larger doses of adenylic acid and ATP block acetylcholine and nicotine pressor effects. This block can be reversed by antiesterases (DFP, prostigmine). It is unlikely that these results are comparable to the stimulation by ATP and adenylic acid of perfused superior cervical ganglion of the cat (FELDBERG AND HEBB, *J. Physiol.* 107: 210, 1948) since the stimulation was obtained in denervated ganglia independently of acetylcholine release at preganglionic nerve ends. However, the results described

and related evidence (see accompanying abstracts and *Federation Proc.* 8: 309, 1949) support their statement on the non-cholinergic (specific) nature of the effects of ATP and adenylic acid.

*Effects of cholinesterase inhibitors on the electrical excitability of the cephalic end of the vago-sympathetic trunk in dogs.* THEODORE KOPPANYI AND ALEXANDER G. KARZMAR\*. Dept. of Pharmacology, Georgetown Univ. School of Medicine, Washington, D. C.

In atropinized animals faradic stimulation of the cephalic end of the vago-sympathetic (CV) produces, in addition to vagal apnea, either purely vasodepressor or only slight pressor effects. Various acetylcholine potentiators (DFP, HETP, TEPP and neostigmine; or adenosinetriphosphate (ATP) and adenylic acid, see the accompanying abstract) either reverse the depressor or considerably increase the pressor effects following CV stimulation. This pressor effect increases with the dose of the agent and may amount to more than 1000% of the original readings. It is not due to a progressive increase of the pressor effects upon repeated stimulation, for in control experiments the initial effect remained constant over long periods of time (6 hours or more). It is, in fact, one of the most dependable effects of acetylcholine potentiators. Large doses of some antiesterases (*Science* 106: 432, 1947) and of ATP and adenylic acid which abolish acetylcholine and nicotine pressor effects in atropinized animals abolished the pressor effects following the CV stimulation. These experiments and the observations that the effect of acetylcholine potentiators on CV stimulation is obtained also with artificial respiration; and that the paralytic doses of nicotine abolish the pressor effects following CV stimulation, but not vagal apnea, prove that the effect is not due to the latter and that it is exerted on the superior cervical ganglia. They confirm our previous communication (*Federation Proc.* 8: 309, 1949) that certain antiesterases potentiate the responses to preganglionic cholinergic nerve stimulations.

*Spectrophotometric studies on whole blood in the red and near infrared regions.* KURT KRAMER, JAMES O. ELAM, GEORGE A. SAXTON AND W. N. ELAM, JR. (introduced by A. ROOS). Dept. of Physiology, USAF, School of Aviation Medicine, Randolph Field, Texas, and Lab. of Applied Thoracic Physiology, Dept. of Surgery, Washington Univ. School of Medicine, St. Louis, Mo.

Beer's law, modified for mixtures of Hb and oxy Hb, can be expressed as follows:  $c_1 = \frac{1}{(\epsilon_2 - \epsilon_1)d} \cdot \ln I/I_0 + \frac{\epsilon_2 C}{\epsilon_2 - \epsilon_1}$ . Where  $c_1$  = Oxygen content of Hb;  $C$  = Oxygen

capacity of Hb;  $\epsilon_1$  = Extinction coefficient of oxy Hb;  $\epsilon_2$  = Extinction coefficient of Hb;  $I/I_0$  = Transmission of light. According to this equation plottings of  $c_1$  against  $\ln I/I_0$  are linear with a constant slope  $1/(\epsilon_2 - \epsilon_1)d$  at any capacity. This has been proved for Hb solutions (1934). In the course of recent efforts to develop an improved two-color oximeter, investigations were carried out to determine the extent to which Beer's law holds for whole blood. The transmission of monochromatic light in the red and near infrared regions through running blood of different oxygen contents and capacities was studied in plane-parallel cuvettes. A straight-line relationship between  $c_1$  and  $\ln I/I_0$  was found at any wave-length, any capacity, and any depth of cuvette, but the slope was not constant at different capacities. The explanation for this could be seen in measurements of light transmission of oxygenated blood at various capacities ( $C_{oxy}$ ). Plottings of  $C_{oxy}$  against  $\ln I/I_0$  showed a considerable increase of absorption ( $\epsilon_1$ ) from 0 to almost 10 vol. % oxygen, leveling off at higher capacities up to 30 vol. %. A similar curve was found for  $C_{reduced}$  though the changes in  $\epsilon_2$  were much less pronounced. Consequently,  $\epsilon_2 - \epsilon_1$  cannot be constant. It is believed that these findings explain most of the errors involved in our conventional procedure with the two-color oximeter, and may point the way towards its improvement in the future.

*Role of carotid sinus reflex during experimental hypertension.* WILLIAM G. KUBICEK, FREDERIC J. KOTKE AND DONNA JEANNE LAKER. Division of Physical Medicine, Univ. of Minnesota Medical School, Minneapolis.

The primary purpose of this investigation was to elucidate the mechanism of the carotid sinus reflex during experimental hypertension. Unipolar shielded silver electrodes were applied to each splanchnic nerve through dorsal incisions. Electrical stimulation 20 hours per day with sinusoidal alternating current of 2 CPS produced an elevation in diastolic blood pressure of approximately 35 mm. Hg. At the end of any given 20-hour stimulation period, the pulse rate was the same as the control level. Cessation of stimulation was followed by a fall in blood pressure within 2-3 minutes. Five minutes after the end of stimulation the blood pressure was found to be within 25 % of normal and the pulse rate increased markedly. These data indicate that after hypertension is established, the carotid sinus and aortic arch reflexes accommodate to the new blood pressure and maintain the pulse rate normal. A sudden decrease in blood pressure toward the control level is opposed by the carotid sinus mechanism which attempts to maintain the blood pressure at the higher level. Two

to three days are necessary for pulse rate and blood pressure to return to normal after the end of several days stimulation of the splanchnic nerves.

*Further observations on thermal conductance of colonic region.* EUGENE M. LANDIS, H. SCARBOROUGH\*, T. B. FERGUSON\* AND R. E. FORSTER, II\*. Dept. of Physiology, Harvard Medical School, Boston, Mass.

The thermal flowmeter previously described by Scarborough *et al.* (*Am. J. Physiol.* 155: 467, 1948) has been calibrated empirically so that changes in thermal conductance of the colonic region itself can be measured. They can then be expressed percentually in terms of control conductance. The thermal conductivity of dead and excised colonic tissue averaged .061 cal.  $\times$  cm.

$\frac{\text{cm.}^2 \times \text{min.} \times ^\circ\text{C.}}{\text{cm.}^2 \times \text{min.} \times ^\circ\text{C.}}$ , i.e., midway between the thermal conductivities of pig muscle and dermis (.066 and .053 respectively, HENRIQUES AND MORITZ, *Am. J. Path.* 23: 531, 1947). In anesthetized dogs atropinization, by reducing peristaltic activity, reduced greatly the variability of the indicated thermal conductance of the colonic region. Hemorrhage a) graded to increase heart rate by 20 beats per minute, b) graded to reduce mean arterial blood pressure to 100 mm. Hg and c) massive and rapid enough to produce profound hypotension reduced thermal conductance of the colonic region by averages of 5, 17 and 27% respectively, including animals with and without atropinization. Re-transfusion increased it by 34%. Larger increases of thermal conductance were observed during and after infusions of Ringer's solution and larger decreases after administering pitressin. The results with pressure breathing were too inconsistent to permit conclusions. The direction and approximate magnitude of possible artefacts have been studied. Calibration directly in terms of volume of colonic blood flow per minute has been attempted but has so far not been possible because of technical difficulties. From the evolutionary standpoint it is interesting that, according to this method of study, the colon with normal circulation can conduct heat away from the mucosal cells at almost exactly the same rate as would free convection by water. By comparison the published figures for average thermal conductance per area of the body surface are much lower than those of the colonic region.

*Respiration of the electric eel.* L. L. LANGLEY (introduced by JOHN BRUHN). Dept. of Physiology, Medical College of Alabama, Birmingham.

The electric eel is an air breather. The gills are poorly formed, there are accessory respiratory organs in the mouth and, if the animal is prevented from reaching the surface of the water to breathe, it will die. On the other hand, so long as the skin is kept moist the animal

can live out of water indefinitely. These observations made it of interest to study the effect of hypoxia and hypercapnia on the respiratory rate. The electric eel, under ordinary conditions, comes to the surface 2 or 3 times per minute to breathe. The air is gulped in, the fish sinks and before coming to the surface again the air is expelled through the gills. CO<sub>2</sub> caused a steadily increasing rate of respiration until a maximum of about 20 per minute was reached at a CO<sub>2</sub> concentration of 5 to 6%. Reducing the O<sub>2</sub> concentration had little effect until it was reduced lower than 12%. The respiration then increased to a peak of 15 per minute. The rate of 20 per minute produced by hypercapnia was never equalled by hypoxia of any degree. In view of the fact that CO<sub>2</sub> has very little effect on the respiration of most fishes these observations are quite striking. In many cases after the normal concentration of O<sub>2</sub> had been restored following severe hypoxia the animal, after taking a breath or two, would not again breathe for periods of 10 minutes or more. Such periods of apnea are also seen under normal conditions. Apparently the electric eel is able to survive, without breathing, for considerable lengths of time.

*Action of sympathomimetic amines on the central nervous system.* ALFRED LEIMDORFER. Dept. of Pharmacology, Loyola Univ., Chicago, Ill.

The effects of the intracisternal (i.c.) injection of various sympathomimetic amines on the central nervous system and on the blood sugar of dogs have been investigated. The injections were made either without preliminary sedatives or when the animals were fully awake following intravenous thiopental anesthesia. The catechol-compounds (epinephrine, isopropyl-arterenol, butanefrine and l-arterenol) exhibit an analgesic, anesthetic and hypnotic action and induce a more or less long lasting central hyperglycemia. The central effects of i.c. epinephrine are the most extensive and the longest lasting. I.c. epinephrine also exerts a stimulation of the respiratory center without changes in blood pressure or electrocardiogram. Isopropyl-arterenol, butanefrine and l-arterenol produce marked changes in the blood pressure and ECG. The phenol-compounds (i.i. synephrine), injected i.c., produce only a short lasting sedation and a moderate, short lasting increase in blood glucose with a high rise in blood pressure and unfavorable disturbances in the ECG. The phenyl-compounds (ephedrine, benzedrine and propadrine), injected i.c., produced no analgesia, no anesthesia, no sleep but great excitement. In addition, a high rise in blood pressure and several disturbances in the ECG, but no changes in the level of the blood glucose follow the i.c. injection of these compounds. The 2-amino heptanes (tuamine and oenethyl) injected i.c. induce no anesthesia, no sleep, but very great excitement, stimu-

lation of motor areas of the cerebral cortex and a high increase in the blood pressure. The blood glucose is only slightly changed or not at all after i.c. injection of these amines. The relationship between chemical structure and the activities exhibited after the i.c. injection of these various amines is discussed.

*Role of component thresholds of cardiovascular response to acetylcholine in a conversion of its vasodepressor into a vasopressor action following anticholinesterases.*  
AMEDEO S. MARRAZZI AND E. ROSS HART\*. Toxicology Section, Medical Division, Army Chemical Center, Md.

With increasing doses of DFP, the vasodepressor response to acetylcholine is first enhanced, then gradually reduced, and finally reversed, i.e., converted to a pressor response. Bradycardia becomes conspicuous with increasing doses and often interferes with the pressor response preventing it or preceding it with a small fall (diphasic response). A vascular rather than a cardiac origin for the pressor effect is thereby indicated. Elimination of the depressor phenomenon suggests that the reversal is due to uncovering a higher threshold pressor effect by block of the lower threshold depressor one, as in the well known similar acetylcholine reversal by atropine. Moreover, if, like the ganglionic, the vasodilator action can be paralyzed by larger doses of acetylcholine or DFP, vasodilator paralysis would precede the ganglionic and in fact occur at a time when DFP enhances cholinergic ganglionic transmission (MARRAZZI AND JARVIK. *Federation Proc.* 6: 354, 1947). At this stage atropine would have little further effect on the blocked vasodilator junctions but could block ganglionic transmission in vasoconstrictor pathways. This is indeed the case for atropine now tends to prevent the pressor effect. The unmasking of a higher threshold ganglionic (including probably adrenal) action of acetylcholine therefore appears to be the cause of this pressor response. Since a preliminary fall in pressure is not necessary, the carotid sinus mechanism does not seem implicated as an essential component. In support of a cholinergic nature for the vasodilator 'paralysis', the reversal can be produced by substituting for DFP repeated injections of the relatively long lasting carbaminoyl- $\beta$ -methyl choline, or the vasodilation reduced by continued acetylcholine infusion.

*Essential factors involved in spreading cortical depression.*  
W. H. MARSHALL, C. F. ESSIG\* AND S. J. DUBROFF\*. National Institute of Mental Health, and Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Md.

It has been established that the spreading cortical depression is secondary to impaired function of the pia-arachnoid system. Dehydration and cooling consequent

on exposing large areas of cortex to room air are crucial factors. Radical internal dehydration of the convoluted brain of the cat makes possible regular elicitation of the reaction by mechanical or electrical stimulation. This report deals with experiments on temperature only as manipulated by changing the temperature of a petrolatum pool over the exposed cortex of rabbits and monkeys. Decreasing the temperature from body level to 15° to 21°C. markedly intensifies the reaction. In many of our experiments with rabbits the distinction is absolute, no spreading depression at body temperature but typical reactions with the oil pool at 17° to 21°C. The monkey experiments show a more decisive difference. The local depression reaction as indicated by the negative voltage change within a few millimeters of the stimulating electrode is always enhanced in the cold. These experimental facts support the hypothesis that spreading depression is due to the incompleteness of a chemical reaction associated with electrical stimulation or mechanical distortion of the pia-arachnoid system and/or cortex. Presumably, the same factors apply to spreading depressions initiated by intense neuronal activity.

*Changes in renal hemodynamics associated with intravenous administration of sodium para-amino hippurate.*  
ROGER K. McDONALD, JOHN H. MILLER\*, NATHAN W. SHOCK AND BENJAMIN MANCHESTER\*. Section on Cardiovascular Diseases and Gerontology, National Institutes of Health, Bethesda, Md., and Baltimore City Hospitals, Baltimore, Md.

The frequent observation that the glomerular filtration rate (GF) is diminished during the measurement of TmpAH suggests significant alterations in renal hemodynamics may be associated with high plasma PAH levels. In a series of 19 subjects the mean GF(CIN) decreased significantly from 88.9 cc/min. at low plasma levels of PAH (1.0-3.4 mg.%) to 77.9 cc/min. at high plasma PAH levels (33-96 mg.%) (TmpAH) ( $t = 4.11$ ;  $p < .01$ ). In 10 subjects the true renal plasma flow (RPF) was measured at low plasma levels and, immediately thereafter, at levels sufficiently high for determining TmpAH. Blood obtained by renal vein catheterization and femoral artery puncture was used for determining the plasma PAH levels. The Fick principle was used in calculating the RPF. The mean RPF increased significantly from 498 cc/min. to 568 cc/min. ( $t = 3.88$ ;  $p < .01$ ), whereas the filtration fraction (FF) decreased significantly from .181 to .144 during the TmpAH periods ( $t = 9.2$ ;  $p < .01$ ). In a second group of 10 individuals the pulse rate and blood pressure were determined at one-minute intervals during and after the intravenous administration of 50 cc. of 20% PAH. Although a significant rise in pulse rate, systolic, diastolic and pulse pressure occurred with the injection of PAH, all values had

returned to pre-injection levels within the 20 minutes preceding the TmpAH determinations. It is concluded that the determination of TmpAH in man is accompanied by an increased RPF and a decreased GF and FF.

*Effect of pooling blood in extremities on cardiac output and renal function in man.* ROBERT L. MCWHORTER, E. HARVEY ESTES, FRANCIS W. FITZHUGH, JR., AND ARTHUR J. MERRILL (introduced by JAMES V. WARREN). Emory Univ. School of Medicine, Atlanta, Ga.

The hemodynamic effects of the application of tourniquets obstructing venous flow have been the object of considerable study. The circulatory alterations so produced are of interest because of their relationship to those seen in postural changes, shock and congestive heart failure. Cardiac output, renal plasma flow, filtration rate, sodium excretion and urine volume were studied in normal subjects before, during and after tourniquets were applied to the lower extremities. The average renal plasma flow value fell to 69% and the average filtration rate value dropped to 87% of control value 31-40 minutes after tourniquet inflation and rapidly returned to control values following release. Sodium excretion and urine volume fell to 56% and 17% of control values, respectively, during this period and were moderately reduced 21-30 minutes following release. The average drop of cardiac output during tourniquet inflation in 9 subjects was 12.5%. Six of 9 subjects had reductions of cardiac output during tourniquets with a rise toward or to control values following tourniquet release. The mechanism of these phenomena is not clear at present. Perhaps a generalized cardiovascular alteration occurs, followed by a compensatory renal response. On the other hand, the primary change may occur in the renal vasculature with a homeostatic reduction of cardiac output. Future work is desired to elucidate further the mechanisms of the alterations observed in these studies.

*A direct study of resonant phenomena of arterial system.*

JOHN P. MEEHAN, JR. (introduced by CHESTER C. HYMAN). Dept. of Physiology, Univ. of Southern California School of Medicine, Los Angeles.

Two types of experiments have been done in an attempt to obtain direct information about the resonant and damping properties of the aorta and large vessels. In the first experiment, pressure contours were simultaneously recorded from the base of the aorta and from the femoral artery. The sudden injection of a small quantity of fluid into the base of the aorta by means of a catheter inserted through a carotid artery served to set up a free oscillation in the aorta. The frequency of

these oscillations and the nature of their transmission to the femoral artery were indicated on the pressure records. The second experiment consisted of introducing a sine pressure wave of any desired frequency at the base of the aorta. Simultaneous pressure records were made at the point where the sine pressure wave was introduced and from the femoral artery. A direct indication of the frequencies best transmitted was obtained by comparing the two simultaneous pressure records. The two experiments outlined were performed on 3 each of the following animals: rabbits, cats, goats and dogs. In all animals tested, the frequency of free oscillations produced in the first experiment was never greater than 60 cycles/sec. Results of the second experiment indicated significant transmission of frequencies above 20 c.p.s. to the femoral artery in only one cat and 2 goats. In the cat, 100% transmission was observed at 33 c.p.s. Frequencies immediately above and below this value were very much damped. Similar results were obtained from the goats.

*Diurnal variations in body temperature.* H. C. MELLETTE AND B. K. HUTT (introduced by STEVEN M. HORVATH). Dept. of Physical Medicine, Univ. of Pennsylvania, Philadelphia.

Continuous rectal temperature determinations were carried out on 11 male and 11 female subjects, all healthy adults, by means of copper-constantan thermocouples and an automatic recording potentiometer. Four 24-hour studies showed that all minimum and almost all maximum temperatures occur between 6 P.M. and 6 A.M., and, therefore, principal emphasis was placed on the night hours. There were a total of 42 such 12-hour observations. Maxima for males averaged 38.32°C. at 8:49 P.M. and for females 38.36°C. at 10:49 P.M.; minima, males 36.83°C. at 6:04 A.M. and females 37.16°C. at 4:48 A.M. With the exception of the levels of the maxima, these values were significantly different between the two sexes. One male showed only 0.70°C. total range throughout the night, while one female showed as much as 2.08°C. Fifteen-minute averages were plotted against time and each graph fitted with the best possible trend line by a modified method of least squares. The mean rate of fall from evening to morning was -0.12°C/hr. for males and -0.06°C/hr. for females, a significant difference. Deviations from each trend line exceeding twice the root mean squares of all deviations for the corresponding sex, were examined for possible relationship to time, evening meal, cold refreshments, activity, falling asleep, awakening, or changes in ambient temperature. Although the inflexions were distributed apparently at random, it was found that of the 12 graphs demonstrating upward inflexions 7 began within 15 minutes of the onset of

moderate activity. In general sleep had a stabilizing effect on the shape of the temperature curve.

*Infrared radiation and olfactory sense.* WALTER R. MILES. Yale Univ., New Haven, Conn.

This paper reports a study bearing on the hypothesis proposed by Dr. Lloyd H. Beck and the author two years ago, that olfaction is stimulated by variations in heat loss from sensory smell receptors, losses that transiently exceed their resting rate of heat radiation. Twenty large American cockroaches (*Periplaneta americana*) were used at a time, and they were placed in the tray an hour before the tests, during which time the tray was in a lighted room and was suspended 30 inches above a homogeneous neutral surface. All the experimental tests were made in a quiet room at night in complete darkness, except for two flash photographs made in each 5-min. testing interval. In conducting the tests the cage was placed successively in 4 different positions of rotation, for 5 min. each (0, 90, 180, and 270 degrees) on a checkerboard surface, so as to provide repeated opportunities for the insects to reorient, that is, make choices in reference to the heat-reflecting and the heat-absorbing surface. A photograph was taken within the first 30 sec. and at the end of the 5-min. period. All the records were photographic. The positions of the numbered insects were examined and tabulated by a technician secured from outside the laboratory who did not know the problem. The records show that in complete darkness there was no prominent tendency for the roaches to cluster together in any corner or area of the cage. The over-all average positive response for the black squares showed 15% greater incidence than for the bright squares. This difference has a critical ratio larger than 5, indicating a probability of less than 1 in 1000 that similar results would occur by chance. The predominating positive response to the black was stronger shortly after cage placement than at the end of 5 min.: 18.4% as compared with 11.8%. This conforms with expectations in reference to olfactory adaptation. Controls were introduced to check on the possibility that the blackened areas were chosen because of some residual odors, even though they had been prepared two years earlier, and this factor appears to have been ruled out. The influence of the factor of a definite ambient odor in the experimental room was also examined. Oil of cloves, which is known to be strongly attractive to roaches, was used. Here the difference favoring the black was 20.8% shortly after cage placement, and 14.2% at the end of 5 minutes. The results of the entire series of tests, as in some other experiments on honeybees, appear to lend support to the general hypothesis that radiation plays a significant part in the physical stimulation of the sense of smell.

*Effect of epinephrine and shock on sodium para-aminohippurate extraction by the rabbit kidney.* FAIRFAX E. MONTAGUE AND FRANK L. WILSON, JR. (introduced by J. V. WARREN). Emory Univ. School of Medicine, Atlanta, Ga.

Trueta *et al.*, using morphologic techniques, have demonstrated a juxtamedullary shunting of blood in the rabbit kidney. We have investigated the effect of this phenomenon on the renal extraction of Na para-aminohippurate. The kidneys were exposed in nembutal-ether anesthetized rabbits. Subcutaneous injections of 250 mg/kg. PAH were given. Simultaneous systemic and renal venous blood samples were taken and compared to determine the percentage extraction of PAH. Epinephrine, 0.1 mg. per injection, was given intravenously; and blood samples taken immediately following visible renal ischemia. Thorotrast contrast medium showed that this ischemia indicated a juxtamedullary shunting of blood. Under these conditions the extraction generally falls to a negative value, 7 out of 9 animals showing a negative extraction. In the 7 the extraction fell to an average of -26.6%, returning to control levels (av. 90.0%) after varying periods of time (10 to 40 min.). Similar falls in PAH extraction were produced by shock. These results show that juxtamedullary shunting of blood in the rabbit kidney, produced by epinephrine and shock, is accompanied by a marked fall in the renal extraction of PAH.

*Influence of high and low oxygen levels on respiratory gas exchange in pulmonary fibrosis and emphysema.*

HURLEY L. MOTLEY. Cardio-Respiratory Laboratory, Barton Memorial Division, Jefferson Hospital, and Dept. of Medicine of Jefferson Medical College, Philadelphia, Pa.

The nature of abnormally high alveolar-arterial oxygen gradients 20 mm. Hg or more (average 28 mm. Hg) was investigated in 55 patients with varying degrees of emphysema and fibrosis by means of low and high levels of oxygenation and intermittent positive pressure breathing (IPPB). The following table shows the effect of variations from air breathing on oxygen transport in the lungs. Arterial pO<sub>2</sub> was determined directly and alveolar pO<sub>2</sub> calculated by the indirect method.

Breathing gas.....	11.5% O <sub>2</sub>	18.7% O <sub>2</sub>	27.8% O <sub>2</sub>	Air IPPB
No. cases.....	27	14	20	14

Inspired pO <sub>2</sub> , mm. Hg	83	-66 <sup>1</sup>	126 <sup>1</sup>	-25	192	+42 <sup>1</sup>	154	+5 <sup>1</sup>
Aeration gradient, mm. Hg (Inspiratory-alveolar pO <sub>2</sub> gradient)	33	-12	50	-7	65	+10	43	-5
Alveolar pO <sub>2</sub> , mm. Hg	50	-54	76	-18	127	+32	111	+10
Transfer gradient, mm. Hg (Alveolar-arterial pO <sub>2</sub> gradient).....	11	-17	21	-10	43	+16	23	-7
Arterial pO <sub>2</sub> , mm. Hg.	39	-37	55	-8	84	+16	88	+17

<sup>1</sup> Change from air breathing.

When the inspired  $pO_2$  was decreased by using low oxygen mixtures, the transfer gradient was decreased, and when high oxygen was used to increase the inspired  $pO_2$ , the transfer gradient was increased. IPPB on compressed air decreased the transfer gradient and increased arterial  $pO_2$  (average 17 mm. Hg). The increase in arterial  $pO_2$  cannot be explained by hyperventilation, for the average alveolar  $pO_2$  was 16 mm. Hg less with IPPB than the 27.8% oxygen. The evidence suggests that IPPB decreases the mean gradient of oxygen tension from alveoli to arterial blood by producing a more uniform alveolar aeration. These studies provided information indicating that in the fibrosis of anthracosilicosis the distribution factor (unequal alveolar aeration and perfusion) is primarily responsible for the increased oxygen gradient between the alveoli and the arterial blood and the diffusion factor with increased resistance of the pulmonary membrane is of minor significance.

*Hemodynamics of renal circulation.* JOHN H. MOYER, HADLEY CONN, KEHL MARKLEY AND CARL F. SCHMIDT. Dept. of Pharmacology, Univ. of Pennsylvania, Philadelphia.

The experiments herein reported were designed so as to test, during electrical stimulation of the sciatic nerve and during adrenalin responses, the functional existence of neurogenically controlled renal by-passes which were reported by Trueta and his associates. The experiments consist of two main types: 1) quantitative studies on changes in renal blood flow (Barcroft-Brodie preparation in 15 rabbits, thermostromuhr in 15 rabbits and bubble flow meter in 15 dogs), systemic blood pressure, and renal arteriovenous differences in blood oxygen content; 2) the injection of india ink into the arterial systems of these same animals. Twenty-one dogs and 55 rabbits were used. Following sciatic nerve stimulation there was a significant and consistent decrease in renal blood flow (34% in dogs and 38% in rabbits after 45 minutes) as compared to controls (4-10%). However, contrary to the observations of Trueta *et al.* the renal venous blood never became arterialized. The oxygen content always decreased and the  $A-RO_2$  increased. The blood pressure rose significantly in about  $\frac{3}{4}$  of the animals. When the cardiovascular system was well preserved following sciatic nerve stimulation india ink was distributed equally throughout the peripheral and the juxtamedullary glomeruli. The 'Trueta by-pass phenomena' (cortical ischemia and subcortical injection) could, however, be reproduced in rabbits but not in dogs following death or following small doses of adrenalin in which cases the action appeared to be local rather than mediated through neurogenic reflexes. From these studies there is no evidence to suggest active patho-physiological renal shunts.

*Production of experimental hypertension in the rat with small adjustable clamps (Goldblatt type) applied to renal arteries.* E. R. MUNNELL\* AND DONALD E. GREGG. Medical Dept., Field Research Laboratory, Fort Knox, Ky.

A small adjustable silver clamp has been developed for application to and constriction of the renal arteries of the rat. The clamp, modeled after the larger Goldblatt clamp, measures 1.7 mm. in height, 1.65 mm. in length, and 1.5 mm. in width. Bilateral application and adjustment of the renal artery clamp at one operation has caused in 10 of 11 rats a persistent elevation of blood pressure. The blood pressure was determined with the photocell method of Kersten *et al.* (*J. Lab. & Clin. Med.* 32: 1090, 1947), as modified in this laboratory. The augmented blood pressure developed in 4-40 days and ranged from 160-220 mm. Hg. In rats operated upon with application and immediate removal of the clamps (controls), pressures ranged from 100-140 mm. Hg. Eight of the 11 rats are alive and well 3-5 months after operation. These studies are being extended.

*A new spectrophotometer adapted to measurements of hemoglobin and oxyhemoglobin in whole hemolyzed blood.* GABRIEL G. NAHAS AND RICHARD C. FOWLER (introduced by WALLACE O. FENN). Dept. of Physiology and Vital Economics, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

A description of an abridged spectrophotometer for measuring hemoglobin and oxyhemoglobin of whole hemolyzed blood is given. Its special features consist of interference filters of narrow transmission band and of a small absorption cell which may be filled anaerobically. Furthermore, it allows for a new, independent standardization in the measurement of blood oxygen saturation. For the mathematical treatment of this method, use is made of the characteristic quotient of two densities of a pigment, established by Lambert-Beer laws of absorption. The formulas derived are a simplification of 'Hufner's Quotient'; this simplification is obtained by determining one optical density at an isobestic point for hemoglobin and oxyhemoglobin (505 millimicrons). An absolute method of calibration for oxyhemoglobin is obtained by applying these formulas to the photoelectric readings. In addition, an empirical calibration was made by the use of the manometric Van Slyke analysis. Twenty-six oxygen saturation measurements by this method and simultaneous determinations by the Van Slyke analysis showed a mean difference of  $-0.04\% \pm$  a standard deviation of 2.2%. In the measurement of hemoglobin, the mean difference between the photometric and the Van Slyke determinations of 23 samples, was  $-0.009$  gm/100 cc.  $\pm$  a standard deviation of 0.25. In the interpretation of

these errors, the role played by white light, plasma turbidity and Van Slyke determinations is discussed.

*Some interrelationships between thyroid and gastric function.* E. S. NASSET AND R. N. WATMAN. Dept. of Physiology and Vital Economics, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Guinea pigs develop perforated peptic ulcers and die in  $135 \pm 20$  hours in response to daily injections of histamine (1 mg/100 gm. body weight/day). Removal of the thyroid glands shortens the survival time to  $56 \pm 8$  hours. Thiouracil treatment does not alter the normal survival time. The administration of thyroxine to the thyroidectomized animal fails to alter the survival time despite the fact that the oxygen consumption is restored to normal. Transplantation of the thyroid glands has no significant effect on normal survival time. It is concluded that the thyroid may influence both the secretion of gastric juice and the resistance of the gastric mucosa to erosion and that this influence is not dependent upon the presence of thyroxine or the normal anatomical connections of the gland.

*Heart rate—breathing rate ratio in birds and mammals.*

EUGENE P. ODUM. Univ. of Georgia, Augusta.

During the course of evolution from stem reptiles the development of the circulatory system in birds and mammals has been largely convergent, whereas the development of respiratory system in the two groups has been divergent, the 'double draft, bellows' system of birds being totally different from 'blind sac, alveolar' system of mammals. A comparative study of heart and breathing rates of a large number of species of small birds and mammals has revealed two differences in the relation of breathing to heart rate which appear to be consistent and to reflect the different respiratory mechanics mentioned above. 1) In birds, the heart rate decreases at or just before the peak of lung and air sac inflation, while in mammals acceleration occurs during inspiration. 2) Small birds and mammals of comparable size have approximately the same basal heart rate but birds breathe less rapidly and more deeply than mammals. In 6 species of birds of less than 150-gram body size the heart rate—breathing rate ratio varied from  $9.1 - 1$  to  $6.9 - 1$ , averaging  $7.4 - 1$ . In 6 species of small mammals of comparable size the ratio varied from  $3.7 - 1$  to  $2.5 - 1$ , averaging  $2.9 - 1$ . Measurements were made with the cardiobromometer, heart and breathing movements being picked up indirectly by means of a piezoelectric crystal, thus making it possible to obtain true basal rates in wild as well as laboratory species.

*Relationship of mast-cells and heparin to foreign proteins.*

NORMAN S. OLSEN AND RALPH O. SMITH\*. Dept. of

Internal Medicine, Washington Univ. School of Medicine, St. Louis, Mo.

In anaphylaxis, peptone shock and acute pneumococcal lymphadenitis in rats it has been noted that the metachromatic (heparin-containing) granules of the mast-cells become dispersed through the surrounding tissues and that the mast-cells become irregular and pale staining. In exploring the cause of the 'mast-cell reaction' by the injection of various materials into the popliteal nodes of rats it was found that of the many substances tested, only foreign proteins, protamines, peptones, turpentine, methylcholanthrene and 2,4-dibenzanthracene elicited the reaction. None of the factors involved in blood coagulation caused any alteration in the nodal mast-cells. *In vitro* studies showed that when proteins causing a 'mast-cell reaction' were buffered to pH below their isoelectric point the addition of small amounts of heparin produced a precipitate which contained both the protein and heparin. This *in vitro* precipitation was not found with compounds not eliciting a 'mast-cell reaction.' Variations in the pH of the reaction, concentration of heparin and foreign protein gave curves characteristic of antigen-antibody precipitin titrations. In addition, specific antibody to the foreign protein added to the washed heparin-protein precipitate caused agglutination of the particles. Thus, the presence of a 'mast-cell reaction' in the popliteal lymph nodes of rats and the *in vitro* precipitation by heparin of antigenic proteins but not of components of the coagulation mechanism, suggests that heparin, in addition to its participation in blood coagulation, may be intimately associated with the mechanisms of immunity.

*Excitability of the mammalian heart during the cardiac cycle: 1. Excitability of the ventricle.* OSCAR ORIAS, CHANDLER MCC. BROOKS, EUSTACE E. SUCKLING, JEROME L. GILBERT AND ARTHUR A. SIEBENS. Dept. of Physiology and Pharmacology, Long Island College of Medicine, Brooklyn, N. Y.

Chlorided silver electrodes were applied to the surface of the exposed right ventricle of dogs under sodium pentobarbital. With the S.A. Node crushed, the heart was driven and its excitability tested with a double impulse electronic stimulator. The rate could be set at will from slightly above spontaneous nodal rhythm about 100 to 250 beats per minute. The second stimulus (square wave), triggered after a delay variable at will following the driving stimulus, was employed to test the excitability. The duration of the testing stimulus could be changed from 0.1 to 13.0 milliseconds and its strength from 0.00 to 30.0 milliamperes. Strength-duration curves were obtained at definite intervals (within 5 msec. error) during the cardiac cycle. Rectal and chest temperature, blood pressure (mercury

manometer) and E.C.G. from standard limb leads were recorded. Forty-two successful experiments were performed in as many dogs. Long duration stimuli (13 msec.) produced responses earlier in the cycle than short duration stimuli (0.1 msec.). A very long latent period was characteristic of the earliest responses. Short cycles showed a shorter absolute refractory period than longer cycles. Following absolute refractoriness, excitability recovery is briefly interrupted by a period of relative inexcitability ('dip') which is particularly well defined for long-duration stimuli. A 'supernormal phase' was never seen. As concerns ventricular excitability, the dog's cardiac cycle is thus divisible into absolute refractoriness, recovery (with the above mentioned 'dip'), and a maximum steady level. The shape of the strength-duration curves and the relationships between them indicate that chronaxie determinations would have been misleading.

*Enzymatic studies on renal tubular secretion of phenol red.*

ROBERT H. PAINTER\*, ETHEL L. WILLIAMS\*, VIRGIL D. WIEBELHAUS\*, S. E. MCKINNEY\* AND KARL H. BEYER. Dept. of Pharmacology, Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

The *in vitro* method of Forster (*Science* 108: 65, 1948) has been modified and adapted to the study of phenol red secretion by the kidney of mammals as well as that of the frog. Within a systematic series of compounds it has been found that their ability to inhibit the tubular secretion of phenol red can be correlated with their structure and is independent of their inherent toxicity (mouse acute I.V. LD<sub>50</sub>). In the frog kidney, the molar ratio of carinamide/phenol red necessary to inhibit completely the secretion of the latter compound is 12.75 and is relatively constant over a range in phenol red concentration of from  $0.026 \times 10^{-3}$  molar to  $0.265 \times 10^{-3}$  molar. Present evidence indicates that oxygen is necessary for the functional integrity of this mechanism. Its utilization apparently proceeds through pathways requiring oxidase, cyanide sensitive (cytochrome) and dehydrogenase components. The ability of the cells to generate high energy phosphate compounds is essential for the maintenance of function of the transport mechanism involved in phenol red secretion.

*Further studies on modification of sensitivity to X-rays by cysteine. I. Time course of protection and comparison of certain related substances.* H. M. PATT, D. E. SMITH, E. B. TYREE\* AND R. L. STRAUBE\*. Argonne National Laboratory, Chicago, Ill.

Cysteine (950 mg./kg. neutralized, I.V.) greatly reduces sensitivity of rats to total body X-irradiation (800 r, 250 kv.) provided that the amino acid is given before the exposure. Comparable protection is obtained

whether cysteine is administered intravenously immediately or 1 hour before irradiation (87% survival of 79 cysteine irradiated; 16% survival of 82 irradiated controls). Injection immediately after exposure or 6 or 24 hours before is without influence. Oral cysteine (1900 mg./kg.) affords significant protection when given 30 to 60 minutes prior to X-irradiation. However, oral administration is not as efficient as intravenous. Cystine, unlike its reduced counterpart cysteine, is not effective by either route. Glutathione, on the other hand, can reduce X-ray toxicity but only when it is given intravenously before exposure. Oral glutathione does not protect rats or mice even though the oral dose is equivalent to the effective oral dose of cysteine in sulfhydryl content. Methionine, ascorbic acid and sodium sulfide do not alter survival after lethal X-irradiation. Results with colloidal sulfur are equivocal and further work is necessary. These findings suggest first that the critical reactions leading to morbidity are complete when the irradiation is terminated and that these reactions are apparently not reversible. Secondly, a sulfhydryl group distinguishes the substances which can protect from those which can not. It is important to bear in mind, however, that all sulfhydryl-containing materials may not necessarily protect and that other reducing substances which are properly distributed, temporally and spatially, may.

*Effects of fever induced by bacterial pyrogen on cerebral circulation and oxygen consumption.* JOHN L. PATTERSON, JR., ALBERT HEYMAN AND FENWICK T. NICHOLS, JR. Depts. of Physiology and Medicine, Emory Univ. School of Medicine, Atlanta, Ga.

Patients with asymptomatic neurosyphilis show only spinal fluid changes indicative of syphilis without other manifestations of the disease. Studies previously reported have shown that patients with this disease have normal values for cerebral blood flow and oxygen consumption. In the present studies fever was produced in 16 such patients by means of the intravenous injection of typhoid vaccine. The cerebral blood flow was determined by the nitrous oxide technique in the afebrile state and during the flush phase of fever in the same patients. Cerebral oxygen consumption was obtained from the product of the blood flow and the arterial-internal jugular venous oxygen difference. Cerebral vascular resistance was calculated by dividing the mean arterial pressure by the cerebral blood flow. The mean elevation of rectal temperature at the time of the determinations was 3.7°F. The mean cerebral blood flow showed almost no change with fever. The cerebral vascular resistance during fever, however, showed a consistent and significant decrease which averaged 25.4%. This decrease tended to be greater with subjects

showing higher values for vascular resistance with normal body temperature. The fall in cerebral vascular resistance was associated with a 24.4% fall in mean arterial pressure. The cerebral blood flow and oxygen consumption values of the patients taken as a group showed almost no correlation in the afebrile state. On the other hand they exhibited a high degree of correlation during the period of elevated body temperature.

*Total hepatectomy in the rat.* FRANCES PAULS (introduced by DOUGLAS R. DRURY). Dept. of Physiology, Univ. of Southern California School of Medicine, Los Angeles.

The extensive use of the rat for metabolic studies has long made a liverless preparation desirable. Total surgical removal of the liver from the rat without impairment of the visceral circulation has not been previously reported. A totally hepatectomized rat has now been prepared by a two stage operation. In the first operation, the portal vein and vena cava are constricted sufficiently to cause development of a collateral circulation. They must not be so constricted as to cause engorgement of the intestinal circulation. It is necessary to place the constricting ligatures in such a way that the mesentery and peritoneum protect the liver lobes from adhering to torn tissue or to the ligature material. If a 100-gram rat is used for the first operation, by the time he has doubled his weight the collateral circulation will be established. During the second operation all lobes of the liver are removed along with the section of vena cava adherent to the right hepatic lobe. Rats which have been totally hepatectomized live as long as 27 hours when given adequate injections of glucose. They are active for several hours, but become comatose before death.

*Pressure pulses and pulse wave velocities in aorta and large vessels of man as determined by direct methods.* L. H. PETERSON, T. G. SCHNABLE, H. F. FITZPATRICK AND H. C. BAZETT. Dept. of Physiology, Univ. of Pennsylvania, Philadelphia.

Small (O.D., 0.9 mm.) flexible, radiopaque plastic catheters of 70 cm. length were inserted through 19 gm. needles into the brachial and femoral arteries of man. Each was then passed into the aorta. The position of the catheters was determined by X-ray and checked with topography. The catheter, capacitance manometer, recording system possessed adequate frequency characteristics (over 70 c.p.s. overdamped). The catheters were then withdrawn in 5 or 10 cm. segments. Pressure pulses and the time difference between pulses of the same origin were observed for apparent pulse wave velocity (PWV) and wave contour. Four such experiments were performed on 3 subjects, ages 26, 33, 64. In all cases there was a net increase in the apparent

PWV as the pulse is propagated toward the periphery. In no case, however, was this acceleration constant. In fact the wave apparently accelerates and decelerates in various portions of both the aorta and the arterial vessels leading to the brachial. In the younger subjects, possessing greater elasticity, these changes in PWV are accompanied by changes in the contour of the pulse. In the older subject the contour remained quite constant throughout the entire length of vessel. The older vessels are tortuous and topographic distances differ from vessel lengths. Various possibilities of the effects of elasticity and tortuosity of vessels are discussed.

*Air flow velocity studies in interpretation of ventilatory reserve.* DONALD F. PROCTOR\*. Dept. of Otolaryngology, Johns Hopkins Medical School, Baltimore Md.

Study of the pneumotachogram during quiet breathing and maximum effort throws some light on the factors limiting ventilatory reserve. Pneumotachographic studies demonstrate that normal individuals are able to reach peak flow velocities in a little over 0.2 second on inspiration and about 0.1 second on expiration. About 0.1 second is needed for the respiratory apparatus to reverse the direction of air flow. Added resistance of 0.5 cm. H<sub>2</sub>O/liter/minute flow results in a 70% reduction in maximum breathing capacity: 0.087 cm. of H<sub>2</sub>O/liter/minute flow causes peak flow velocities, 60% of those with a resistance of 0.013 cm. H<sub>2</sub>O/liter/minute flow. Studies in patients with pulmonary disease with decreased ventilatory reserve frequently show inability to reach high flow velocities. The patterns of flow during maximum effort are abnormal.

*Effects of environmental heat stress and exercise on renal blood flow and filtration rate.* LEO R. RADIGAN AND SID ROBINSON. Dept. of Physiology, Indiana Univ., Bloomington.

Renal plasma flow and glomerular filtration rate were determined on 5 normal male subjects at rest and during moderate exercise on the treadmill (3 m.p.h. up a 5% grade) in both a cool (21°C.) and a hot environment (50°C.). Sodium para-aminohippurate and mannitol respectively were used for the determination. In the cool environment the mean renal plasma flow of the resting men was 695 cc/minute and it dropped 42% during exercise in the same environment. At rest in the hot environment the mean renal plasma flow was 426 cc/minute. Exercise in the heat caused a decrease of 36% from the resting level. In the cool environment the glomerular filtration rate of the men at rest averaged 108 cc/minute and exercise in the environment did not significantly alter it. In the heat the average filtration rate of the resting subjects fell to 84 cc/minute and

when they exercised there was a further decrease to 70 cc/minute.

*Effect of stretch upon action potential of voluntary muscle.* H. J. RALSTON, E. W. WRIGHT\*, B. FEINSTEIN\* AND V. T. INMAN\*. College of Physicians and Surgeons of San Francisco and Univ. of California Medical School, San Francisco.

Several investigators have described an increase in the amplitude of the action potential of frog voluntary muscle when the muscle is stretched. Forbes, Ray and Hopkins (*Am. J. Physiol.* 65: 300, 1923) observed the effect in the frog gastrocnemius but not in the sartorius, and attempted to explain the discrepancy upon the basis of the geometrical arrangement of the fibers in the gastrocnemius. Fulton (*Proc. R.S. s. B.* 97: 406, 1925) observed the effect, however, in both the gastrocnemius and sartorius, and concluded that there is an intimate relationship between electrical response and tension. The present investigators show that the contradiction in the results with the sartorius was due to the fact that the muscle was stimulated directly in one case, and indirectly in the other. Explanation of the effect is provided by the direct observation that more fibers of the sartorius contract upon stretch when the muscle is being stimulated directly by single submaximal stimuli. It is also shown that an increase in the amplitude of the action potential occurs in an unstretched muscle during indirect tetanic stimulation, but not in a stretched muscle. This effect is due to the participation of more muscle fibers as developed tension increases. Certain possible implications of the preceding results for normal muscle contraction are discussed.

*Mechanism of muscular fatigue in adrenalectomized animals.* E. RAMEY, M. S. GOLDSTEIN AND RACHMIEL LEVINE. Dept. of Metabolic and Endocrine Research, Medical Research Institute, Michael Reese Hospital, Chicago, Ill.

It is well known that adrenalectomized animals exhibit rapid fatigability on effort of the skeletal musculature. The causes of this fatigue may lie in disturbed function or metabolism of the muscle itself, the neuromuscular apparatus, or in the proper environment necessary for continued muscular work (e.g. adjustment of circulation etc.). We should like to report some experiments designed to narrow the above possibilities in order to make it possible to study the more intimate mechanisms of this fatigue and its relief by the C-11 oxysteroids. When adrenalectomized rats, maintained in good health by saline, were subjected to a swimming test, they showed general and muscular collapse within 10 to 20 minutes. Normal controls swam for at least 3 hours. Muscle strips (diaphragm and abdominal wall)

taken from similarly treated adrenalectomized rats were stimulated electrically while immersed in an oxygenated nutrient solution (inductorium at the rate of 6/min.). In contrast to the fatigue exhibited *in vivo*, such muscles showed the same capacity and durability as did control strips taken from normal rats. The lack of abnormal fatigue *in vitro* was shown even by muscle strips removed from adrenalectomized animals in a state of exhaustion after swimming. It appears, therefore, that the causes of the rapid fatigability of adrenalectomized animals should be looked for in the environmental factors supporting muscular work such as circulatory and/or neurological adjustments, rather than in any intrinsic dysfunction of the muscle cell.

*A theory of the formation of HCl by the stomach.* WARREN S. REHR. Dept. of Physiology, Univ. of Louisville School of Medicine, Louisville, Ky.

In previously reported experiments it has been found (*Am. J. Physiol.* 144: 115, 1945) that the production of  $H^+$  can be controlled in the secreting stomach by the application of electric current across the stomach. When current is sent from serosa to mucosa  $H^+$  production is increased and when sent in the opposite direction  $H^+$  production is decreased. Analysis of these data reveals that the production of  $H^+$  can be increased by the application of less electrical energy, capable of doing useful work, than the minimum amount necessary to produce the increased HCl when this is calculated as the minimum amount necessary to separate an isotonic HCl solution from a solution of the same ionic composition as that of plasma. Assuming that the flow of electric current controls the  $H^+$  production by a process basically similar to the formation of  $H^+$  at a metal-solution junction, then the EMF's of the stomach would have to deliver a given amount of current for the production of a given number of  $H^+$ . Evidence indicates that the EMF's of the stomach can deliver this amount of current. Primarily on the basis of these findings a theory of HCl formation has been formulated, according to which the EMF's of the mucosa send electric current across the canalicular border of the parietal cells in the direction of the canaliculi, and this flow of current in the presence of a gastric stimulant results in  $H^+$  secretion. The return flow of current is responsible for the transport of the  $Cl^-$ .

*Release of gastrin in response to bathing pyloric mucosa with acetylcholine solution.* C. R. ROBERTSON, CLEMENT MARTIN, GEORGE SLEZAK AND M. I. GROSSMAN. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

Parasympathomimetic drugs are well known stimulants of gastric secretion but the mechanism of their action is not known. There are at least three possi-

bilities: a) direct stimulation of the parietal cell, b) release of gastrin which in turn stimulates the parietal cell and c) potentiation of other stimulants. In the present study, perfusion of 0.5% solution of acetylcholine through isolated pyloric pouches of dogs caused an acid response in the fundic pouch. Pepsin secretion was not stimulated. When a similar amount of acetylcholine was administered orally, subcutaneously, rectally, or perfused in the fundic pouch, gastric acid or pepsin secretions were not stimulated. Because a) the juice did not have high pepsin content characteristic of parasympathomimetic stimulation, and b) other routes of administration of acetylcholine did not cause acid secretion, we conclude that acetylcholine stimulates gastric secretion by releasing gastrin from the pyloric mucosa. Nicotine in the pyloric pouch did not stimulate secretion which indicates that acetylcholine acts by its muscarinic action. Since direct perfusion in the fundic pouch or intraarterial injections of acetylcholine (Morton and Stavsky, 1949) do not stimulate secretion, this rules out the possibility of direct parietal cell stimulation by acetylcholine. This does not alter the possibility that acetylcholine acts directly on the parietal cell to potentiate histamine (Robertson and Grossman, 1948).

*Some factors affecting salt exchange of men exposed to heat.* SID ROBINSON. Dept. of Physiology, Indiana Univ., Bloomington.

Men, unacclimatized to heat, were exposed to daily work in a hot environment (50°C. with 18% humidity). Data on their salt exchange confirm previous reports that unacclimatized men performing daily work in the heat and sweating large volumes undergo a rapid reduction in urinary chloride output and may show a more gradual decrease in the concentration of chloride in the sweat. Results of this study indicate that the reduction of sweat chloride under these circumstances depends upon the development of a salt deficit by the subjects during the first days of exposure. 1) The reduction occurred gradually in 3 to 6 days when 4 to 7 liters of sweat were secreted daily by unacclimatized men consuming 50 to 170 mEq. of NaCl per day. In these cases the output of salt in the sweat during the first 2 or 3 days exceeded the intake and a chloride deficit was developed. 2) The reduction did not occur when men consuming 170 mEq. of NaCl per day were exposed to the same stress for shorter periods (two hours or less) so that the total chloride output in the sweat was considerably lower than the intake. 3) The response was reversed by raising the salt intake to exceed the output of men who, in daily exposures to heat, had previously reduced the chloride concentration in their sweat to low levels following the develop-

ment of a salt deficit. The sweat chloride began to rise in these men on the third day after the intake was increased even though the same exposures were continued and their daily sweat output remained the same. The rise occurred in spite of improved temperature regulation and lower body temperature.

*Effects of pyruvic acid on oxygen consumption of rat liver homogenate.* TRUE W. ROBINSON. Yellow Springs, Ohio.

In previous work using the Warburg technic to study the respiration of various tissues, particularly mouse sarcoma, it was found that small concentrations of indole acetic acid stimulated oxygen consumption of the sarcoma and large concentrations inhibited it. It was of interest therefore to determine if a normal cellular substrate like pyruvic acid would also inhibit in large concentrations. If so, this would throw some light on the behavior of cellular metabolism under low oxygen tensions. The oxygen consumption of rat liver suspension obtained from the Potter homogenizer decreases rapidly both in control and experimental vessels. This decrease is exponential and a logarithmic plot of the oxygen quotient against time results in a straight line over most of the range. However, since the first portion is curved it can be determined that the oxygen consumption is controlled by at least two factors. The first controls the major component of the oxygen utilization but its effect has largely disappeared after one hour. Since the rate of oxygen consumption decays exponentially with time the mean life or time constant can be obtained. By means of the time constant a true comparison can be made between the rates of oxygen consumption of control and experimental vessels. Using this technic it was determined that the oxygen consumption was increased by pyruvic acid concentrations of about  $10^{-3}$  molar and inhibited by these greater than  $10^{-2}$  molar. Thus it may be possible that high concentrations of pyruvic acid accumulating in the cell may act autocatalytically to inhibit oxygen consumption.

*Mechanism of pressor response to increased intracranial pressure.* SIMON ROXBARD. Cardiovascular Dept., Medical Research Institute, Michael Reese Hospital, Chicago, Ill.

The pressor response to increased intracranial pressure has been attributed to an anemia, anoxia or asphyxia of the brain resulting from the increased resistance of flow of blood into the cranium. We have carried out experiments which show that the pressor response appears to depend on other mechanisms. Thus, severe anoxia produced by 100% nitrogen breathing or asphyxia produced by clamping the trachea

produce only a limited increase in blood pressure. During the pressor response to asphyxia an increase in intracranial pressure produces a new and much greater rise in blood pressure. In the chick the blood pressure response is almost exactly equal to the increase in intracranial pressure even though the control blood pressure is not even approached. The rate of blood pressure rise is a function of the degree of the intracranial pressure stimulus. Momentary increases in intracranial pressure produce an increase in blood pressure equivalent to the stimulus. Cocainization of the brain surface blocks the response to increased intracranial pressure but does not inhibit the response to asphyxia. The production of a negative intracranial pressure results in a fall in blood pressure. These data can be explained by assuming the presence of an intracranial baroreceptor similar to that of the carotid sinus. By virtue of its position, such an intracranial baroreceptor could act as a differential manometer registering the difference between the intravascular and the intracranial pressures. When this difference decreases, vasoconstriction ensues. When the difference is increased, as occurs with decreased intracranial pressure, the vasomotor center is stimulated to produce a vasodilation.

*Growth of organs in relation to vitamin A deficiency.*

STELIOS C. SAMARAS, NICHOLAS DIETZ, JR. AND DANIEL J. HINGERTY (introduced by CHARLES M. WILHELMJ). Dept. of Biological Chemistry and Nutrition, Creighton Univ. School of Medicine, Omaha, Nebr.

The interrelationships between vitamins and hormones, and their effects on growth are as yet insufficiently understood; much further work is desirable for a more complete understanding of factors influencing normal and pathological growth. To study the role of vitamin A in organ growth, the following investigations were carried out, using 150 male albino rats under normal feeding conditions and after varying periods of vitamin A deprivation. The ratio of organ weight to body weight was determined for a number of groups of normal and deficient animals, ranging in age from 4-14 weeks; the influence of carotene administration on weight changes was also studied.

From the results it appears that all organs are not affected equally by the lack of the vitamin; of the organs studied (liver, lung, spleen, heart, kidney, testis, thyroid) the greatest effects on growth retardation were noted in the liver and testis. Liver weights of control animals, 7, 11, and 14 weeks old, (expressed as percentage of total body weight) were 4.7, 3.9, 3.9, respectively; the percentages for deficient animals of corresponding ages but maintained on vitamin A-deficient diet from the age of 4 weeks were 3.9, 2.9,

2.8. In the control animals the corresponding percentages for the testis were 1.27, 1.15, 1.33; in the deficient animals the percentages were 1.33, 0.92, 0.99. Oral administration of carotene in the early stages of the deficiency was effective in producing an acceleration of growth in these organs so that they approached the normal values for the age group.

*Vitamin A metabolism in normal and deficient rats.*

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To determine the rat's ability to utilize carotene when in various stages of vitamin A deficiency, 10 of 40 male white rats, 4 weeks old, were fed the regular Purina diet; the remainder were maintained for various periods (4 to 10 weeks) on a vitamin A-deficient diet, after which they received orally a single dose (400 U.S.P. units) of carotene. The normally fed animals steadily built up vitamin A stores in liver with no accumulation of carotene and when 12-14 weeks old had: carotene: liver 2.3  $\mu\text{g/gm.}$ , intestine 3.5, lung 3.1; vitamin A: liver 85.1, intestine 1.5, lung 1.8. Rats 4 weeks deficient with liver vitamin A stores (as indicated by control analyses) less than 1  $\mu\text{g/gm.}$ , but no symptoms other than weight loss showed an immediate return to normal after carotene with appreciable storage of vitamin A one week later (carotene: liver 3.8  $\mu\text{g/gm.}$ , intestine 2.1, lung 2.0; vitamin A: liver 9.1, intestine 1.2, lung 0.9). Rats with more advanced deficiency and mild eye symptoms also showed striking amelioration lasting 2-5 weeks, after which they again began to show symptoms and on autopsy had no measurable quantity of vitamin A in any organ. Very severely deficient rats with marked hemorrhagic conjunctivitis were unable to utilize carotene, dying shortly after its administration, despite the presence of appreciable carotene quantities in liver (8.1  $\mu\text{g/gm.}$ ), intestine (12.6), and lung (7.3). The animals apparently first develop reversible biochemical and functional derangements followed later by irreversible histological alterations impairing carotene utilization.

*Carotene conversion in rats: role of reticulo endothelial system (RES).* STELIOS C. SAMARAS AND DANIEL J. HINGERTY (introduced by CHARLES M. WILHELMJ). Dept. of Biological Chemistry and Nutrition, Creighton Univ. School of Medicine, Omaha, Neb.

The common role of the RES and vitamin A in combating infection suggested the following investigation. Forty-four male white rats, 4 weeks old, were divided into two groups, one group deprived of vitamin A until liver stores were decreased to less than 1  $\mu\text{g/gm.}$ , the remainder receiving a normal diet for the same

period. Half of the animals in each group were injected intraperitoneally with 2 cc. 2% trypanblue in saline; 18 hours later, all the animals received orally 0.1 cc. of carotene concentrate (670 U.S.P. units). The animals were killed after 2, 4 and 24 hours and carotene and vitamin A contents of liver, intestine and lung estimated by spectrophotometric methods. After carotene administration liver vitamin A stores increased by the following average amounts: normal rats uninjected 9  $\mu\text{g/gm.}$ , injected: 30  $\mu\text{g/gm.}$ ; deficient rats uninjected: 2.1  $\mu\text{g/gm.}$ ; injected 0.9  $\mu\text{g/gm.}$  Conclusions: 1) Carotene conversion takes place within 2 hours of its administration; 2) the ability of the vitamin A-deficient rat to convert carotene is markedly diminished; 3) injection of trypanblue increased the ability of the normal rat to convert carotene given 18 hours later but in the deficient rat it retards the conversion. Apparently, the trypanblue stimulated the RES in the normal animals, whereas in the deficient animals, whose resistance was already decreased, the RES was further deteriorated causing further depletion of the already deficient organism.

*Electrical measurements on squid giant axon by complex attenuation method.* OTTO H. SCHMITT, PETER A. STEWART AND VIOLA E. SCHMITT. Depts. of Zoology and Physics, Univ. of Minnesota, Minneapolis.

It is possible to show on simple theoretical grounds that a sinusoidal stimulus applied to a nerve between two ringlet electrodes should produce a signal in the extrapolar regions which decays in amplitude exponentially and which changes in phase linearly with distance. It is further possible to show that these two attenuation constants contain all the data needed beside longitudinal nerve resistances to determine quantitatively at each test frequency the characteristic impedance, membrane conductance, membrane susceptance, and phase velocity of propagation along the nerve. These predictions are extremely well borne out by experiment and permit verification of previous measurements. Considerable new information also is emerging regarding the effects of drugs and ions. For squid nerve a membrane capacitance of 1-1.5 mfd/cm.<sup>2</sup> is uniformly found at 500-1000 c.p.s. Conductance for normal nerve in the 200 c.p.s. region is about 1 milliom/cm.<sup>2</sup> but in the mid-frequency region around 500 c.p.s. total conductance of the membrane drops to near zero and may even dip into the negative region under the influence of calcium. A strong reactive component in the 75-300 c.p.s. region is found which is very sensitive to drugs and which can be pushed far into the positive susceptance region, especially by calcium and veratrine. It is also possible to demonstrate progressive changes in the membrane and response to

drugs long after the nerve has ceased to respond and after conductivity of the membrane has increased many-fold. Because the method is not limited to single fiber preparations and does not require giant axons, it is foreseen as a valuable means for determining electrical characteristics of nerve preparations unsuitable for single fiber study and its extension to muscle experimentation seems feasible. An electronic-mechanical computer which gathers the necessary impedance data quickly and reduces it to graphical form immediately has been built and has greatly facilitated this work.

*Impedance of vertebrate nerve fibers before and during passage of an impulse.* GORDON M. SCHOEFFLE AND NOAH SUSMAN\*. Dept. of Physiology, Washington Univ. School of Medicine, St. Louis, Mo.

Direct current threshold stimuli of various durations were applied to axone bundles or to whole frog sciatic nerve minus perineural sheath. In such structurally simplified systems threshold is attained when the electrotonic potential at the cathode reaches a critical value regardless of its time rate of change. Dichotomous splitting of the nerve, or even removal of the sheath alone, endows the system with properties that more nearly approach those of an ideal situation in which a single fiber is surrounded by a very thin fluid film. The mathematics of cable theory then predicts that membrane voltage must attain a critical value over a small region in order to fire off the fiber. The membrane resistance in fibers prior to excitation appears to be unaffected by currents of rheobasic magnitude. The linearity of the system finds further confirmation in the observation that changes in single fiber spike height are proportional to applied steady state polarizing voltage. A decline of electrotonic potential during activity is indicative of the predicted fall in membrane resistance at this time. Hence it is logical to conclude that change in spike height during anodal or cathodal polarization merely involves a transient shift in the steady state or drop across the membrane due to the fall in resistance alone and not alterations in the distribution of electromotive forces giving rise to the action potential proper.

*Mechanism for renal clearance of sodium in the dog: effect of decreased and increased load on reabsorptive mechanism.* EWALD E. SELKURT AND ROBERT S. POST\*. Dept. of Physiology, Western Reserve Univ. Medical School, Cleveland, Ohio.

Previous investigation has revealed that as sodium load to the tubular reabsorptive mechanism is decreased by reduction in glomerular filtration, reabsorption becomes complete and excretion diminishes to the vanishing point. This has been confirmed by additional experiments. The apparent dependence of tubular reabsorption on load prompted investigation of the effects

of increased loads by rapid infusion of 5% NaCl. In all cases, marked excretion of sodium followed as the reabsorptive mechanism became saturated. In some animals, the amount reabsorbed appeared to reach a constant, limiting value (range, 6.3 to 12.5 mM/min.), but in others the amount reabsorbed continued to increase gradually as load was further increased. When load was increased by infusion of isotonic saline (2.0 to 3.85 l. total), a similar marked increase in excretion of sodium was noted. Increased load here resulted from increase in filtration rate. Even though the efficiency of reabsorption was impaired, the relationship of reabsorption to load always showed an increasing slope. Constant plasma sodium in this series ruled out the possibility that increased secretion of ADH had modified the reabsorptive mechanism, and strongly suggested that the tubular mechanism for reabsorption of sodium is limited to the extent that the kinetics of reabsorption are impaired as excessive loads are delivered to the tubular cells.

*Plasma exchange rate and volume of distribution of radio-sulfate.* GUY C. SHEATZ\* AND WALTER S. WILDE. Dept. of Physiology, Tulane Univ. School of Medicine, New Orleans, La.

By diffusion, in contrast to filtration, the large hydrated sulfate ion should cross a capillary membrane more slowly than sodium. To test this we construct plasma concentration-time curves after instantaneous intravenous injection of Na-24 and S-35 labelled sulfate into rats. Of the S-35 counts added to plasma,  $93.7 \pm 7.5\%$  is recovered as benzidine sulfate with carrier. In contrast to earlier chemical studies tracer sulfate moves instantly into erythrocytes slightly exceeding the chloride ratio even early in the time course of the plasma curve. We fit an exponential equation which describes the exchange of blood sulfate from corpuscles-plasma, as a common pool, across the capillaries. Each minute 0.11 of the blood sulfate exchanges. This is 0.15 of plasma sulfate, whereas the plasma turnover for sodium is 0.17. The similarity of the latter argues for a filtration transfer mechanism. Our radiosulfate space for muscle works out to be 12%, as for chloride, but smaller than for Na-24. This does not fit a pore size limitation theory for anions. An intracellular phase in liver for sulfate is indicated by a space of 33.7%, as compared to 18.4 for Na-24 and 20.0 for chemical chloride. Sulfate space for the entire rat of 39.9% became 34.2 when corrected for urine tracer and for plasma recovery loss. Na-24 space is 25.4%. The exchange into bone, cartilage, mucoprotein, etc., must account for the apparent failure of radio-sulfate to represent a strictly extracellular pool.

*Sodium and potassium exchange between cells and plasma of mammalian blood.* C. W. SHEPPARD AND W. R. MARTIN. Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Studies of cation exchange by the isotope method (*Federation Proc.* 8: 145, 1949) have been extended to include potassium and sodium exchange in erythrocytes of man, sheep, cow, and dog. The following exchange rates were obtained:

SPECIES	CELLULAR CONC. mmol./l.		K EX- CHANGE RATE IN % CELL- ULAR K/HR.	Na EX- CHANGE RATE IN % CELL- ULAR Na/HR.
	K	Na		
Man.....	91	11	1.8	13.6
Cow.....	25	70	3.8	16
Sheep.....	12	82	2.0	~5
Dog.....	5.5	106	1.0	12.7

The potassium exchange rate for canine cells is corrected for processes of exchange with the white cell fraction (*Biol. Bull.* 95: 287, 1948). Although the cells with a high concentration of a cation tend to exchange that ion rapidly and low cellular concentration is associated with a correspondingly low exchange rate, a general rule cannot be established without further investigation. Determinations of potassium exchange in human blood at different temperatures gave a  $Q_{10}$  of 2.35. The rate of loss exceeds the rate of uptake below 15°C. Studies were also made of exchange in human blood in which potassium ions were substituted for part of the sodium in the plasma. Increasing the potassium concentration to as much as four times normal has no significant effect on the potassium exchange rate. The results are suggestive of processes controlled more by intrinsic cellular factors rather than by strictly physical transport phenomena.

*Acute changes in blood flow through the coronary circuit following retrograde perfusion of coronary sinus.* R. E. SHIPLEY AND R. S. STUDY\*. Lilly Laboratory for Clinical Research, General Hospital, Indianapolis, Ind.

The recently proposed operation for arterialization of the coronary sinus has been reported to be effective in preventing or reducing infarction following occlusion of a main coronary branch (Roberts, Beck, Stenstrom). Although it has been presumed that the operation increases the flow of oxygenated blood through the myocardium, no direct flow studies have been reported to substantiate this belief. In 7 dog experiments left coronary inflow and coronary sinus outflow were measured with optically recording rotameters along with the arterial blood pressure. Autoperfusion of the

coronary sinus with arterial blood (at the dog's existing BP) consistently caused a moderate to marked fall in left coronary inflow and BP. In all experiments the procedure caused greater reductions of coronary inflow and BP than those resulting from occlusion of the coronary sinus. Even when blood was allowed to flow from the left coronary artery during perfusion of the coronary sinus, myocardial blood flow was not adequate for the maintenance of the blood pressure. These findings indicate that if arterialization of the coronary sinus protects the heart against subsequent coronary occlusion, such protection is not attributable to an immediately augmented flow of blood to the myocardium, but occurs in spite of an initially decreased blood flow. The mechanism by which the operation prevents or reduces infarction was not determined and deserves further investigation.

*Effect of intragastric feeding on voluntary food intake.*

GEORGE C. SLEZAK\* AND M. I. GROSSMAN. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

Two dogs provided with simple gastric fistulas were standardized by allowing them to feed *ad libitum* for 45 minutes at the same hour each day. When the voluntary intake remained constant for at least 2 weeks, intragastric feeding was initiated. Measured amounts of the same food as was used in the *ad libitum* feeding were introduced daily through the gastric fistula from 6 to 20 hours before the *ad libitum* feeding. Intragastric feedings equivalent to 30%, 50% and 125% of the control level of voluntary intake were performed and continued for periods of one to 5 weeks. The intragastric feeding of 30% of the control level of voluntary intake produced no sustained drop in voluntary intake or significant change in body weight. The intragastric feedings of 50% and 125% produced no significant change in body weight but were compensated for by a proportionate drop in voluntary food intake. Daily intragastric feedings of 50% produced after 3 weeks a 50% reduction in voluntary food intake. Daily intragastric feedings of 125% produced after one week an 80% reduction in voluntary food intake. In all cases no drop in voluntary food intake appeared until after the second day of intragastric feeding. This would indicate that there must be a certain degree of storage of the added nourishment before the adjustment mechanism is put into action. Since oral, gastric and intestinal factors can be shown not to operate under the conditions of these experiments, the existence of tissue repletion factors in regulating food intake is indicated.

*Modification of sensitivity to X-rays by cysteine. II. X-ray cysteine dosage relationships.* D. E. SMITH, H. M.

PATT, E. B. TYREE\* AND R. L. STRAUBE\*. Argonne National Laboratory, Chicago, Ill.

The purpose of this work was to determine the quantitative aspects of the protective action of cysteine against X-irradiation. Sprague-Dawley rats were injected with neutralized cysteine (dosage range 350 to 950 mg/kg.) immediately before total-body irradiation with 250 kv. X-rays (dosage range 600-1600 r). Animals were irradiated in pairs, one rat having been injected via the tail vein with cysteine and the other with an equivalent volume of a 5% NaCl solution. Survival at 30 days post-irradiation was the criterion for cysteine protection. At an X-ray dosage of 800 r (killing an average of 80% of the controls) and cysteine dosages of 350, 475, 575, 875 and 950 mg/kg. the protection afforded by cysteine was roughly proportional to the dosage. Experiments employing cysteine dosages of 475 or 950 mg/kg. and irradiations of 600, 700, 800, 1000, 1200, 1400 and 1600 r revealed: 1) Above 800 r cysteine protection decreased as the amount of irradiation was increased, 2) above 800 r the higher cysteine dosage afforded 13 to 20% greater protection than the lower one; 3) 2.9 to 4.75 mg/kg. of cysteine were required to protect against each r above the just sublethal dosage; 4) the LD<sub>50</sub> X-ray dosage was raised from 725 r for the controls to about 1060 r and 1220 r by the low and high cysteine dosages respectively.

*Further observations on local action of epinephrine on human sweat glands.* R. R. SONNENSCHN, HENRY KOBRIN AND M. I. GROSSMAN. Dept. of Clinical Science, Univ. of Illinois College of Medicine, Chicago.

Intradermal injection into the forearm of 0.05 or 0.1 cc. epinephrine-HCl, 1:1000 to 1:1,000,000, produced a definite local sweat response in 26 of 34 normal subjects, tested by Randall's iodine-starch paper method. Similar effects were produced by neosynephrine and arterenol. No sexual or racial differences were apparent. The response commenced within 2 minutes after injection and lasted up to 2 hours; it closely approximated the area of vasoconstriction. In 12 subjects, injection into the palm failed to cause sweating, except in one case. Preliminary intradermal injection of atropine 1:10,000 or 1:100,000, or TEA 1:100 had no effect on the epinephrine response; dibenamine and ergotamine caused marked inhibition; procaine 1:100 caused slight to marked inhibition. In several subjects who were sweating spontaneously, after the initial stimulation by epinephrine, the local area failed to manifest spontaneous activity for one to more than 24 hours. Sweating caused by epinephrine is not secondary to vasoconstriction because 1) in 3 experiments, ischemia from arterial tourniquet caused only a gradual decrease in

spontaneous sweating; 2) in 8 experiments, after a passive hyperemia had been produced by a venous tourniquet, epinephrine caused only a minimal vasoconstriction, but the sweat response was unaltered. No conclusion can yet be drawn as to the physiological significance of these findings.

*Effects of renal artery-nerve stimulation on renal blood flow measured directly, and by clearance and extraction of diodrast.* R. S. STUDY\* AND R. E. SHIPLEY. Lilly Laboratory for Clinical Research, General Hospital, Indianapolis, Ind.

A study has been made in dogs comparing the directly measured renal venous blood flow (measured with a rotameter) with renal blood flow as calculated by the clearance and extraction percentage of diodrast. The direct and calculated renal blood flow values checked closely only during the control periods and only if the absolute amount of diodrast removed from systemic plasma was used in calculating the blood flow (indirect). During renal artery-nerve stimulation, with low frequency alternating current the directly measured renal blood flow and urine flow decreased. The calculated renal blood flow decreased much more than the actual renal blood flow because urine flow decreased markedly and most of the diodrast which had been filtered and secreted during the stimulation period had not yet reached the renal pelvis where it would be obtainable for analysis. The direct and calculated blood flows approximated each other after one or two periods of recovery when the diodrast which was filtered and secreted during the period of stimulation was washed out by the returning urine flow. The extraction percentage of diodrast and inulin from plasma decreased significantly during renal artery-nerve stimulation and followed the direct renal blood flow. Under the conditions of the experiment and with the type of stimulation used the intra-arterial injection of trypan blue showed diffuse renal ischemia with patchy, pyramidal areas of injection without a preponderance of dye in the juxta-medullary areas.

*Changes in visual searching performance following cerebral lesions.* HANS-LUKAS TEUBER\*, WILLIAM S. BATTERSBY\* AND MORRIS B. BENDER. Psychophysiological Lab., Dept. of Neurology, New York Univ. College of Medicine, New York City.

Disturbances in visual searching behavior after cerebral lesions have been observed in man and in the experimental monkey. The following investigation was undertaken to establish whether disturbances in visual searching in man are necessarily associated with visual field defect or motor disturbance due to cerebral lesions. The subjects were 12 brain injured children, 14 brain injured adults and 26 normal controls (matched for age). Six test fields were constructed consisting each of

48 test patterns (capital letters, geometric figures) distributed irregularly over a square surface. Each test field was projected on a rear-projection screen so that it subtended 60 by 60 degrees. In the center of the screen duplicates of one of the 48 test figures were projected, one at a time. In 48 timed trials (8 for each field), the subject had to find as quickly as possible that figure in the periphery which was identical with the figure shown in the center. Results show significantly prolonged searching times in the entire field. This occurred in 10 of the 12 brain injured children, and in 8 of the 14 brain injured adults. Among these, 7 children and 4 adults showed searching performances which were asymmetric, i.e. particularly slow in homonymous half fields or quadrants. Slowing in searching performance may occur in the absence of either perimetric field defects or cerebral motor disturbances, or both.

*Opacity and dimensional changes in stimulated crayfish nerve.* JULIAN M. TOBIAS AND SIDNEY SOLOMON. Dept. of Physiology, Univ. of Chicago, Chicago, Ill.

A structural moiety which, it would seem, must coexist with chemical and electrical events to make up the phenomenon of excitation has been sought for in polarized crayfish nerve. Crayfish nerve has been used because it contains relatively little myelin. Polarizing currents have been used because otherwise fleeting events might be accumulated thereby. Using currents of 0.2-500 microamperes one can produce an increase in opacity and shrinkage of the nerve anodally with an increase in transparency and swelling cathodally. This change is detectable by eye and easily quantified with a photometer. The phenomenon can also be produced by rectified tetanizing stimulation and therefore must outlast the current producing it. The change is reversible, spontaneously and slowly on simply interrupting the current, more rapidly upon reversal of the current. Boiling or soaking the nerve in chloroform make the change unobtainable. Soaking in 0.2 M KCl does not prevent it but soaking in 0.16 M CaCl<sub>2</sub> does. The phenomenon involves the axon per se. It is not due to some change in intercellular material though this may participate. It is suggested that the underlying mechanism may consist of anodal agglomeration of colloid with dehydration and cathodal solation with hydration. Water may be moved electroendosmotically along the long axis of the nerve toward the cathode.

*Effect of convulsant and anticonvulsant agents on activity of oxalacetic carboxylase.* CLARA TORDA AND HAROLD G. WOLFF. New York Hospital, Kingsbridge Hospital (V.A.), and the Depts. of Medicine and Psychiatry, Cornell Univ. Med. College, New York City.

The concentration of carbon dioxide (and therefore bicarbonates) available for metabolic processes in the

brain may be an essential factor in the induction and prevention of convulsive seizures according to Lennox and collaborators. The transportation of inorganic bicarbonates and the processes of carbon dioxide fixation are important mechanisms for the regulation of the concentration of carbon dioxide in the tissues. Results obtained through investigation of inorganic bicarbonate transportation activated by carbonic anhydrase suggest an accumulation of the bicarbonates in the presence of convulsant agents, and a decrease of concentration of tissue bicarbonates in the presence of anticonvulsant agents. In the following, the effect of certain convulsant and anticonvulsant agents on the activity of oxalacetic carboxylase was investigated to ascertain whether these agents exert any effect on carbon dioxide fixation by dicarboxylic acids. The activity of oxalacetic carboxylase was inhibited in the presence of the convulsant agents used (acetylcholine, caffeine, camphor, cocaine, dichlorodiphenyltrichloroethane, methylsalicylate, pentamethylene tetrazol, picrotoxin, scilliroside, and strychnine) in concentrations of  $1 \times 10^{-3}$  M and less, the greatest inhibition averaging 75%. The activity of oxalacetic carboxylase was somewhat increased in the presence of the anticonvulsant agents used (hydantoin, methylphenyl-ethyl hydantoin, phenylhydantoin sodium, and phenobarbital), the greatest increase of activity averaging 30%. The activity of oxalacetic carboxylase was not modified by tridione and some other agents that do not induce or prevent convulsive seizures. These results suggest a further accumulation of bicarbonates in the tissues in the presence of convulsant agents due to inhibition of at least one essential process of carbon dioxide fixation.

*Effect of some enzyme poisons on in vitro respiration of brains of rats of various ages.* DAVID B. TYLER. Dept. of Embryology, Carnegie Institution of Washington, Baltimore, Md.

The  $O_2$  uptake of excised brain tissue in response to  $NaN_3$  ( $3.0$  to  $500 \times 10^{-4}$  M) and to 2,4-dinitrophenol ( $0.1$  to  $100 \times 10^{-6}$  M) has been investigated. Using Ringer-prepared homogenates it is found that the brain of the newborn is more sensitive to the inhibitory effects of azide in all concentrations studied than that of the adult. With chopped brain the optimum concentration of dinitrophenol (DNP) producing augmentation is lower for the newborn than for the adult; however, in the newborn higher concentrations of DNP are required to produce first evidence of inhibition of respiration. In attempts to localize these actions of DNP, experiments were made using differently prepared homogenates and employing different substrates. When brains were homogenized in distilled water for 5 minutes and the concentration was then adjusted to that of Ringer glucose phosphate (RGP) or reinforced

with various coenzymes, carriers and transport mechanisms, the response to various concentrations of DNP was only inhibition. Reducing the time to one minute resulted in a slight response to the augmenting concentrations. On the other hand, homogenates prepared in RGP or in isotonic media that preserved nuclear structure responded to the various concentrations of DNP as did chopped brain. Microscopic examination of those homogenates that were only inhibited by DNP revealed no, or only very few, normal appearing nuclei, and the results indicate a relationship between the number of such nuclei and the response to the augmenting effect of DNP. Using Ringer-homogenized adult brain, augmentation of from 80 to 150% was produced by DNP ( $5.0 \times 10^{-5}$  M) with either glucose, lactate or pyruvate as the substrate, the latter freshly prepared as the Na salt from doubly distilled acid and slowly neutralized in the cold. With substrates such as citrate, fumarate, malate or succinate only inhibition in varying degrees occurred. Inhibition of respiration by  $5.0 \times 10^{-5}$  M DNP was found when hexose diphosphate (2 exper.) or the Cori ester (1 exper.) were used as substrates. Such concentrations of DNP increase the  $O_2$  uptake with glucose over 150%.

*Effect of ambient air temperatures on temperatures in respiratory tract.* EDWIN G. VAIL\* and FRED A. HITCHCOCK. Laboratory of Aviation Physiology and Medicine, Ohio State Univ. Columbus, Ohio.

Previously reported experiments on dogs show that a temperature gradient exists in the respiratory tract. When the ambient air temperature is  $25^\circ\text{C}$ . the average temperatures are as follows: trachea during inspiration,  $35.7^\circ\text{C}$ .; trachea during expiration,  $36.9^\circ\text{C}$ .; and bronchioles,  $37.5^\circ\text{C}$ . The rectal temperature is slightly higher than the deep lung temperature. Similar experiments have been carried out in which anesthetized animals have been exposed for 30 minutes to ambient air temperatures of  $-3$  to  $-16^\circ\text{C}$ . Average values obtained in 12 experiments in which tracheal and deep lung temperatures were measured, show that the temperature of inspired air was decreased  $6.1^\circ$ , of expired air  $4.9^\circ$ , and the temperature of the deep lungs  $3.6^\circ\text{C}$ . In two experiments the exposure to cold caused a drop of  $3.4^\circ$  in rectal temperature and of  $2.6^\circ$  in deep tracheal temperature. In 3 experiments in which the animals breathed through an endo-tracheal tube the drops in temperature resulting from exposure to cold were: inspired air,  $7.6^\circ$ , expired air  $6.2^\circ$ , and in the deep lungs,  $3.3^\circ$ . After removal of the endo-tracheal tube the temperature decreases were: inspired air,  $2.9^\circ$ , expired air,  $2.7^\circ$  and deep lungs,  $1.6^\circ\text{C}$ . In all experiments at the termination of 30 minutes cold exposure, the animals were decompressed to a terminal pressure of 68 mm. Hg in 8 seconds. After the decompression, effective respiration stopped and the tracheal temperatures rose

slightly in 8 of the 12 experiments. No significant change in the temperature of the deep lung occurred.

*Effects of hypothermia on the potentiation induced by tetanus and treppe in rat muscle.* SHEPPARD M. WALKER. Dept. of Physiology, Washington Univ. School of Medicine, St. Louis, Mo.

The potentiation of tension in response II of 2 twitches summated, which occurs in normal muscle, is absent in hypothermic muscle (*Federation Proc.* 8: 161, 1949). The view, that remaining internal forces of response I account for the enhancement of tension in response II of two twitches summated, was tested by application of quick stretch during isolated twitch responses of the rat triceps surae *in situ*. Doubt was thrown upon this view by the findings in normal muscle that quick stretch and ordinary lengthening produce similar effects on twitch tension. The extensibility of the muscle increased during contraction to a maximum at the peak of the twitch and decreased during the falling phase to the value existing before the twitch. Normal and hypothermic muscles were then examined for potentiation of twitch response by procedures designed to eliminate or minimize differences in the mechanical state of the muscles. Neither tetanus nor treppe produced such potentiation in hypothermic muscle, although both induced marked enhancement in the same muscle before cooling. These findings suggest that the potentiation of tension in the second of two twitches summated is due, at least in part, to chemical changes occurring during the first.

*Rate of exchange of potassium in plasma, liver and muscle after intravenous tracer.* W. GORDON WALKER\* AND WALTER S. WILDE. Dept. of Physiology, Tulane Univ., New Orleans, La.

We analyze whether exchange of potassium in plasma, liver and muscle is represented by equations describing exchange between simple homogeneous compartments. K-42 as KCl free of Na-24 is injected in 7 seconds into the ear vein of nembutalized rabbits. The transcapillary phase of the concentration-time curve for plasma is so rapid that points are not obtained. For the first 50 minutes the best fit is  $a^* = 0.94e^{-0.1t} + 0.14$ , where  $a^*$  is counts/ml. plasma/counts injected/gm. body weight. The concentration at zero time,  $a^*_0 = 1.08$ , of the apparent volume of tracer undergoing biologic decay, implies an early distribution, not in plasma, but in total body water. However, the concentration is not found by direct measurement, even in the large bulk of muscle water at any early time. Thus while we cannot localize any discreet mass of potassium as undergoing exchange, we can say that its turnover is  $0.94 \times 2.1/1.08 = 0.087/\text{minute}$  and is evidently an exchange

into tissue elements. Fenn, Noonan, Mullings and Haeger report rapid entry of K\* into liver with specific activity reaching and remaining above plasma for long periods. We note late continuing gain in calculated specific activity while plasma is falling. This is impossible for a single mixed pool no matter how fast the liver ingests K\* during early plasma enrichment. There must be multiple pools possibly including accumulation in one-way channels, as bile capillaries. After a peak, liver K\* falls coincident with an elevation in plasma at 50 minutes. K\* accumulates slowly and steadily in muscle. Liver and muscle turnover rates are about 0.05 and 0.001 per minute.

*Use of spring-lever and strain gauge equipment in determining contractile force of heart muscle in open-chest and closed-chest mammalian experiments* (Demonstration). R. P. WALTON, O. J. BRODIE, J. S. LEARY, M. DEV. COTTEN AND P. C. GAZES (introduced by T. G. BERNTHAL). Dept. of Pharmacology, Medical College of South Carolina, Charleston.

The Cushny myocardiograph levers typically attached to the right ventricle in open-chest mammalian preparations have been modified by the addition of coiled metal springs operating against the thrust of the levers. The spring tension necessary to bring the levers to stand-still is expressed in grams and taken as a measure of the isometric systolic tension (IST). In order to estimate the influence exerted by various circulatory factors on these measurements, a series of experiments have been carried out in which these various factors have been found to vary in degrees which quantitatively are slight to moderate in terms of the marked changes produced by drugs. Experiments include the effects of size changes, occlusion of great vessels, massive saline infusions, hemorrhage, rate changes and coronary occlusion. General agreement with the Starling principle is obtained by mechanical stretching of the muscle segment to approximately twice its length, which increases contractile force to levels several times greater than in the control periods. Experiments with saline infusions and with hemorrhage also appear to demonstrate the classic principle but are less consistent. Various groups of drugs have been characterized in terms of IST changes; these include the digitaloids, sympathomimetic amines, veratrine alkaloids, dicumarol and dinitrophenol. The procedure of measurement has been further modified by the substitution of strain gauge equipment for the coiled metal springs. Various adaptations of the strain gauge principle have been used, some of which, because of greater compactness, are suitable for use in chronic, mammalian experiments. In some experiments, typical measurements have been made for periods up to 3 weeks postoperatively.

*Projection of vestibular nerve to cerebral cortex of the cat.*

EDWARD M. WALZL AND VERNON MOUNTCASTLE.

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The projection of vestibular sensation to the cerebral cortex has been investigated by an application of the evoked potential technique. Peripheral excitation was accomplished by either of two methods: 1) brief mechanical stimuli were delivered through minute fenestrations to the vestibular end organs or 2) the vestibular nerve, exposed distal to its juncture with the cochlear nerve by microdissection, was stimulated electrically. Such stimuli evoke initially positive, nearly monophasic potential changes in a small, sharply circumscribed area of the cerebral cortex of the cat. The projection to the cortex is principally contralateral, but stimulation of the ipsilateral nerve activates a part of the same region. We have not demonstrated the specific localization in the cerebral cortex of particular end organs. The area lies above and anterior to the anterior descending limb of the suprasylvian sulcus and includes the cortex of its anterior bank. It borders the auditory areas posteriorly, the first somatic area anteriorly, and the second somatic area ventrally. Vestibular stimulation evokes no potential changes in other regions of the lateral surface of the hemisphere. The potentials evoked by electrical stimulation of the vestibular nerve have a latency of 6-8 msec. duration. They are most readily observed under very light barbiturate narcosis, and disappear with deepening anesthesia at levels permitting the activation of visual, auditory, or tactile regions of the cortex by appropriate physiological stimuli.

*Micro-cine photographic demonstration of bubbles in frogs rapidly decompressed from a range of 7 to 10 atmospheres to ground level.*

LOUISE WARNER AND ALBERT R. BEHNKE (introduced by MELVIN H. KNISELY). National Naval Medical Center, Naval Medical Research Institute, Bethesda, Md., and Dept. of Anatomy, Medical College of South Carolina, Charleston.

It has been known for many years that men and animals rapidly decompressed from pressures experienced by divers *a)* develop the syndrome known as divers' bends and *b)* develop bubbles in their bodies. It has not been known whether all or most bubbles were inside vessels or in tissue spaces and extravascular fluid, nor precisely how the presence of bubbles initiates the signs and symptoms of bends. In 50 frogs and 20 guinea pigs decompressed from pressures of 7 to 10 atmospheres to ground level in 60 seconds or less, the tissues were transilluminated with fused quartz rods, and studied with microscopes; great numbers of bubbles were found passing through vessels of 1-2

mm. in diameter and embolizing much smaller vessels. Relatively few bubbles have been found outside vessels in tissues. The chambers of the heart have occasionally been filled with a bubbly froth which the contracting heart did not push forward. Currently it seems probable that major damage is done by the plugging of vessels. Studies are continuing with improving techniques aimed at learning the condition of pressure and rates of decompression which determine and limit the rate of bubble formation and the rates of bubble reabsorption.

*A technique for visualization of experimental peptic ulcer formation in the dog.* R. N. WATMAN AND E. S. NASSET. Dept. of Physiology and Vital Economics, Univ. of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The Mann-Williamson dog, although classic in peptic ulcer studies, has certain inherent disadvantages, namely, 1) difficulty in maintaining a good nutritive state; 2) unfeasibility of determining accurately the time of occurrence and course of the ulcer except by repeated laparotomy; and 3) the lesion differs from human stoma ulcer because of deviation of the duodenal contents from the upper intestine. A method is presented based on anastomosis of gastric pouches to selected intestinal loops combined with a Maydl-type enterostomy which has the following advantages: 1) consistent production of peptic ulceration in the selected ones; 2) maintenance of excellent nutrition; 3) the ulcer area can be isolated from the intestinal stream to a greater or lesser degree at will; 4) the causative agent is uncontaminated gastric juice from a pre-selected gastric pouch of any desired size, type and location; 5) the lesion is easily visualized using a standard infant-size proctoscope. The method was applied to three dogs using Thomas type fundic pouches. It was successful in all cases. The pathology simulated human subacute and chronic peptic ulceration with fibrous proliferation.

Factors affecting the time of appearance and course of the ulcer are: 1) size, location and type of gastric pouch; 2) level of intestine selected; 3) size of the stoma of the pouch-intestine anastomosis; 4) distance from the pouch-intestine anastomosis to the entero-anastomosis; 5) distance from external intestinal button to the pouch-intestine anastomosis.

*A method for estimating the uptake of  $P^{32}$  by cellular tissues of the body in man: The effect of insulin.*

RICHARD L. WECHSLER, HILDA KLOTZ, AND SEYMOUR S. KERY. Dept. of Physiology and Pharmacology, Univ. of Pennsylvania Graduate School of Medicine, and Diabetic Coma Project, Philadelphia General Hospital.

Because of the importance of phosphorylating mechanisms in carbohydrate metabolism and therefore diabetes, it was considered of interest to develop a method for measuring the uptake of  $P^{32}$  by the cellular tissues of the body in man. The problem could be solved by injecting, along with  $P^{32}$ , a substance which would distribute itself in the extracellular regions and have negligible uptake by the cellular regions of the body. Thiocyanate ion is convenient and has been used extensively as a measure of extracellular fluid volume. The difference between the plasma disappearance curves of the two substances ( $SCN^-$  and  $P^{32}$ ) was considered an approximation to the uptake of  $P^{32}$  by body cells. Control studies were carried out on 6 male schizophrenics with no known organic disease. As a means of testing the method, 5 of the control patients were given various doses of insulin. The method showed that in every case insulin increased the cellular uptake of  $P^{32}$  at each time interval. The mean cellular uptake, expressed as percentage of injected tagged phosphate, was before insulin 33.7, 50.2, 58.8, and 68.4% at 30, 60, 90, and 180 minutes respectively. After insulin, corresponding results were 47.8, 75.5, 84.9, 89.9%. These increases were all statistically significant. This method could be applied to any ion, indeed to any injected substance whose identity could be maintained in the blood stream.

*Research applications of thermistors.* ERWIN K. WEISE AND TRUE W. ROBINSON. Univ. of Illinois, Urbana, and Yellow Springs, Ohio.

In measuring techniques thermistors are coming more and more into general use. Their unique features open new possibilities for quicker, simpler and more sensitive electrical measurements. Those who wish to use thermistors in their research work may not be familiar either with the limitations or the advantages of thermistors. The experiments demonstrate one of the major features of the thermistors; namely the very high sensitivity of an electrically heated thermistor to variations of cooling by a streaming gas. A thermistor assembled in the air stream moving in and out of the mouthpiece of a breathing mask can record the breathing cycle in detail by variations of the current flowing through the thermistor if a constant voltage is applied. A hot wire requires a more sensitive bridge circuit than does the thermistor. This is demonstrated. A glass tube which is sealed by a finger placed in one end and which has the other end connected with a capillary (a thermistor is assembled in the axis) can convert the volume oscillations of the finger due to the blood pulse wave into variations of the air stream cooling the thermistor. By this means the pulse wave of the peripheral circulation can be picked up. If the glass tube is interchanged with a little funnel which is placed on the carotid sinus or artery the beat of the heart can be readily recorded.

The successive steps in production of thermistor threads of a diameter of about  $90\mu$  is demonstrated by samples.

*Influence of induced hypo- and hyperthyroidism on vitamin E requirement of chicks.* ROBERT S. WHEELER AND JESSE D. PERKINSON, JR. Poultry Dept., Univ. of Georgia, Augusta, and Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.

One hundred two, one-day-old White Leghorn male chicks were fed an E deficient diet and an equal number were fed the same diet with adequate vitamin E. Thirty-four chicks from each group were made hyperthyroid and 34 were made hypothyroid. The total mortality from E deficiency was highest in the hyperthyroid animals, intermediate in the euthyroid, and lowest in the hypothyroid. The mean age at death was 15, 20 and 28 days respectively. Forty-eight % of the hyperthyroid chicks were dead before any of the other animals showed deficiency symptoms and 35% of the euthyroid chicks were dead by the time any of the hypothyroid chicks died.

*Variability of effects of growth hormone preparations on renal functions.* H. L. WHITE, PETER HEINBECKER AND DORIS ROLF\*. Depts. of Physiology and Surgery, Washington Univ. School of Medicine, St. Louis, Mo.

We have reported (*Am. J. Physiol.* 157: 47, 1949) great increases in PAH and inulin clearances in normal and hypophysectomized dogs on growth hormone administration. Our material, prepared by the Wilhelmi-Fishman-Russell procedure, has been generously supplied by Armour's Research Laboratories. A second (Armour 3PKS3R) and a third (Armour H1902) preparation have had no effect on these clearances or on oxygen consumption in normal dogs, while producing large increases in clearances and in oxygen consumption in hypophysectomized dogs, with no increase in cardiac output. The difference between the active and inactive (on normal dogs) preparations is presumably not due to a higher thyrotrophin contamination of the active preparation (Armour 3PKR3), since its thyrotrophin assay is essentially the same as H1902, and 0.5 mg/kg/day of 3PKR3 was effective in normal dogs, while up to 1.4 mg/kg/day of H1902 was ineffective. Electrophoretic studies of the various preparations, carried out at Armour's Research Laboratories, have not enabled a prediction as to what preparation will be active. The hypophysectomized dog is more sensitive to the unidentified active principle than is the normal.

*Mechanism of reduction of acid by normal human duodenum.* C. M. WILHELMJ, ADOLPH SACHS\*, BEN SLUTZKY\*, AND ANTHONY BARAK\*. Depts. of Physiology and Medicine, Creighton Univ. School of Medicine, Omaha, Nebr.

A triple lumen, double balloon tube was used. The duodenum was blocked just below the pyloric sphincter and at a point approximately 10 inches below this. An hydrochloric acid solution (approximately 0.117 normal) containing phenol red was instilled into the duodenum between the balloons. Routinely 100 cc. of acid was left in for 5 minutes, then withdrawn and another 100 cc. introduced until 5 to 7 samples were obtained. The percentage of phenol red, total chloride, neutral chloride and volume were determined in each sample. In most instances more than 50% of the reduction in acidity was due to dilution, the remainder to neutralization. The mean composition of the mixed duodenal secretions in 100 samples from 4 normal subjects was: 364 mg. chloride per cent; normality 0.089; total acid reducing power (dilution plus neutralization) expressed as normality, 0.212. The average amount of secretion per 100 cc. of sample was 45 cc. In some experiments the normality of the secretions decreased as the experiment continued, in others it remained practically constant; the amount of secretion usually increased. The maximal reduction in acidity occurred in 5 minutes and there was only a small additional decrease in 10 minutes. In 2 minutes the reduction was only approximately one half that of the 5-minute period. Nausea without vomiting lowered the acid reducing capacity of the duodenum.

*Nature of peripheral resistance.* ARNOLD H. WILLIAMS\* AND HENRY A. SCHROEDER. Hypertension Division, Dept. of Internal Medicine, Washington Univ. School of Medicine, St. Louis, Mo.

The nature and distribution of peripheral resistance in both normal and hypertensive man is an important subject. It is necessary to establish the relative contribution of each of the various regional resistances to blood pressure in order to evaluate peripheral resistance as a whole. This is a preliminary report of the method used and of the role of the resistance of the limbs in normotensive and hypertensive subjects. Pressure cuffs were inflated rapidly and simultaneously about both thighs and the left arm (which approximates one-sixth of the circulation) to stimulate maximal vasoconstriction of these extremities while blood pressure was measured directly in the right brachial artery and cardiac output with a ballistocardiograph. The psychic element was evaluated by venous occlusion. Suprasystolic compression elevated the pressure of normotensives about 5% and of hypertensives about 7%. Venous occlusion produced about the same change. Considerably greater increases of pressure would have resulted if total peripheral resistance had not decreased by 12% in the normotensive and 36% in the hypertensive groups. The observed 5% decrease of cardiac output did not account for the difference. Thus the limbs

play a relatively minor role in the maintenance of blood pressure as their maximal vasoconstriction results in little change of pressure.

*A single-scale absolute reading ear oximeter* (Demonstration). EARL H. WOOD, (with the technical assistance of LUCILLE CRONIN), Mayo Foundation, Univ. of Minnesota, Rochester.

Two modified oximeter earpieces were calibrated on the basis of their responses on empirical optical filters with the use of the single-scale circuit described by Wood and Geraci (*J. Lab. & Clin. Med.* 34: 387, 1949). Earpiece 1 was used to determine the oxygen saturation of arterial blood in 33 ears of 18 normal subjects. Thirteen were white and 5 were Negroes. Ages ranged from 2 to 50 years. The average saturation obtained when the subjects were breathing air was  $96.7 \pm 0.3$  (93-101)% and  $99.4 \pm 0.4$  (94-106)% when they were breathing oxygen. Earpiece 2 was used on 24 ears of 17 normal subjects. Twelve were white, 2 were Chinese and 3 were Negroes. The average saturation of arterial blood was  $96.7 \pm 0.3$  (94-100) and  $99.7 \pm 0.5$  (96-104)% when the subjects were breathing air and oxygen, respectively. Eighteen simultaneous Van Slyke and oximetric determinations of oxygen saturation of arterial blood were made concerning 6 patients with congenital heart disease during supine rest, breathing air or oxygen, standing and walking. The percentage of oxygen saturation of arterial blood by Van Slyke analysis of radial artery blood averaged 85 and ranged from 39 to 100. The standard deviation of the differences between simultaneous Van Slyke and ear oximeter determinations in these hypoxemic patients was 4%. The instrument can be operated by alternating current or battery, is compact, relatively inexpensive, simple to operate and apparently is accurate enough to warrant its use for most clinical estimations of oxygen saturation of arterial blood.

*A comparison of oximetric measurements on histaminized and heat-flushed ears.* EARL H. WOOD, JULIAN R. B. KNUTSON AND BOWEN E. TAYLOR. Mayo Foundation, Univ. of Minnesota, Rochester.

Arterial saturation was recorded simultaneously from both ears of 12 subjects as the mixture breathed was changed from air to 40% O<sub>2</sub>, from 40 to 99.6% O<sub>2</sub>, from air to 99.6% O<sub>2</sub> and during rapid intravenous injection of methylene blue (1 mg/kg.). One of the ears was flushed by the heat of the earpiece alone, while the other ear was previously subjected to histamine iontophoresis at 4 milliamperes for 1.5 minutes each on the dorsal and ventral skin surfaces of the pinna. The optical density of blood in the heat-flushed ear ( $\log [IR_0/IR_B]$ ) averaged 0.12, 0.14 and  $0.17 \pm 0.01$  after the earpiece had been on the ear approximately 1, 10 and 60 min-

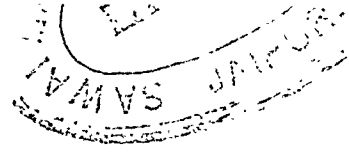
utes, respectively. Equivalent values for histaminized ears were  $0.25$ ,  $0.26$  and  $0.28 \pm 0.02$ . The average indicated oxygen saturations of blood in the ears at these times were  $96.3 \pm 0.8$ ,  $97.7 \pm 0.3$  and  $98.6 \pm 0.6\%$  for the heat-flushed ears and  $96.4 \pm 0.4$ ,  $96.8 \pm 0.3$  and  $97.2 \pm 0.5$  for the histamine-flushed ears, respectively. Average arterial saturations obtained when the subjects were breathing air,  $40\% \text{ O}_2$  and  $99.6\% \text{ O}_2$  were  $98.5 \pm 0.2$ ,  $100.3 \pm 0.3$  and  $100.8 \pm 0.2$ , respectively, for the heat-flushed ear and  $97.3 \pm 0.2$ ,  $98.7 \pm 0.3$  and  $99.2 \pm 0.2$  for the histamine-flushed ear. The lung-to-ear circulation time after inspiration of oxygen was  $4.7 \pm 0.3$  and  $5.0 \pm 0.4$  second, and the arterial equilibration time  $55 \pm 5$  and  $61 \pm 8$  seconds at the two ears. After injection of methylene blue the decrease in transmission of red light by the ear (arm-to-ear circulation time) began at  $13.4 \pm 1$  and  $13.5 \pm 1$  second, while the maximal decrease occurred at  $26.8 \pm 1.8$  and  $26.4 \pm 1.9$  seconds at the heat-flushed and histamine-flushed ears, respectively. Apparently, the additional flushing of the ear produced by histamine iontophoresis does not materially affect the accuracy of ear oximetry.

*Blood pressure of normal dogs observed over a twelve-month period.* JOHN A. ZAPP, JR. (introduced by D. B. DILL).

Haskell Laboratory of Industrial Toxicology, E. I. du Pont de Nemours and Company, Wilmington, Del.

The blood pressure of dogs was measured indirectly by means of a pediatric size cuff applied to the upper part of the hind leg, a mercury sphygmomanometer, and a stethoscope for auscultation over the saphenous artery. Six adult male dogs were used for a study of the variability in blood pressure over an extended period. Systolic and diastolic pressures were measured twice daily, 5 days a week for a period of 14 months. The first 2 months were considered to be a training period and the last 12 months were used for the study in variability. At the end of the study the means of 4 consecutive readings were plotted as quality control charts with limit lines at plus and minus 3 standard errors of the mean (estimated from the average range of the groups of four). Three of the 6 dogs were handled by the same technician over the entire period. A change of technicians was made during the observation period of the other 3 dogs. The results indicate that there is sometimes a fall in blood pressure during the training period. There was no indication of seasonal trends, but other trends were observed in some dogs.

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